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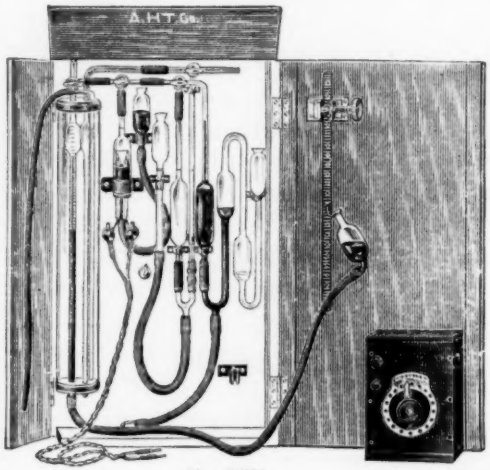

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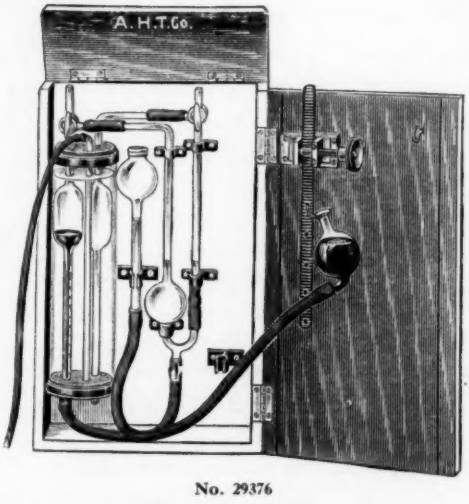

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CONDUCTIVITY AS A MEASURE OF PERMEABILITY.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, October 17, 1918.)

The method of electrical conductivity has been used by the writer to investigate the permeability of protoplasm. In this work it has been assumed that we can measure the permeability

CORRECTION.

On page 97, Vol. XXXVI, No. 1, October, 1918, line 13 of the second paragraph, following the parenthesis insert *25 cc. of 20 per cent sodium carbonate.*

substance and observing whether the conductivity continued to show the marked changes which it undergoes in the living tissue under the influence of certain reagents such as NaCl and $CaCl_2$. The results of such experiments show that after killing the cells the conductivity no longer changes when NaCl and $CaCl_2$ are applied, but the question still remains whether the killing agent has not in some way altered the intercellular substance, so that its conductivity no longer changes as before.

In the case of *Laminaria* (which furnished the material for the experiments which have hitherto been described) the question could not be settled as completely as was desirable because the chemical behavior of the intercellular substance was not sufficiently known. Therefore it seemed desirable to repeat the experiments

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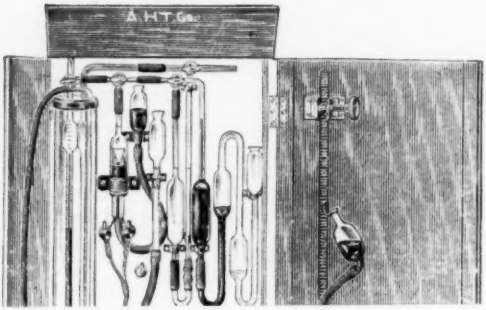

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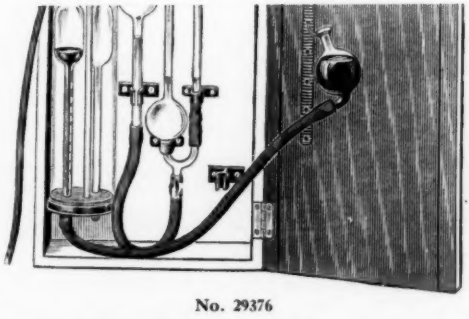

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The method of electrical conductivity has been used by the writer to investigate the permeability of protoplasm. In this work it has been assumed that we can measure the permeability of protoplasm by determining the electrical conductivity of the tissues. But it may be objected that what we investigate by this method is not the protoplasm but only the non-living intercellular material by which the masses of protoplasm (cells) are separated in the tissue.

It is obvious that when a current passes through the tissue a considerable part of it must flow in the intercellular substance. Since this has a much lower resistance than the living protoplasm, the question arises whether any of the current passes through the protoplasm.

It might appear that this question could be settled by killing the protoplasm with agents which do not affect the intercellular substance and observing whether the conductivity continued to show the marked changes which it undergoes in the living tissue under the influence of certain reagents such as NaCl and $CaCl_2$. The results of such experiments show that after killing the cells the conductivity no longer changes when NaCl and $CaCl_2$ are applied, but the question still remains whether the killing agent has not in some way altered the intercellular substance, so that its conductivity no longer changes as before.

In the case of *Laminaria* (which furnished the material for the experiments which have hitherto been described) the question could not be settled as completely as was desirable because the chemical behavior of the intercellular substance was not sufficiently known. Therefore it seemed desirable to repeat the experiments

with plants whose intercellular substance consists of cellulose and to kill the tissues by means which are known to produce no irreversible changes in the properties of cellulose. For this purpose experiments were made with a green marine alga (*Ulva*) and with a marine flowering plant (*Zostera*) both of which have cellulose walls.

The means employed for killing were various, including partial drying, treatment for a few hours with 25 per cent alcohol, exposure for a few minutes to sea water at 40°C., as well as placing for several hours in a solution of NaCl of the same conductivity as the sea water.

All these methods of killing produced similar results in that the conductivity rose to a constant value (indicating death) and was not thereafter affected by exposure to reagents which produce great alterations in the conductivity of living tissue.

We must therefore conclude that the alterations of conductivity observed in living tissue are due to changes in the protoplasm and not to changes in the non-living intercellular substance.

A very striking proof of this consists in adding to the sea water solid $CaCl_2$ (or a strong solution of $CaCl_2$). This greatly increases the conductivity of the solution and consequently of the intercellular substance which is freely imbibed with the solution. It might therefore be expected that the conductivity of the tissue would be increased. Just the contrary is the case. Although the conductivity of the intercellular substances increases, this is more than offset by the decrease of conductivity of the protoplasm due to the direct action of the calcium on the living protoplasm. The result is a decrease of the conductivity of the tissue as a whole. This decrease does not occur with tissue killed by the agents described above.

Another striking piece of evidence is found in the fact that the temperature coefficient of the electrical conductivity of living tissue differs from that of dead tissue. This could not be the case if the living protoplasm did not conduct a part of the current.

We must therefore conclude that while a part of the current flows in the intercellular substance another part flows through the protoplasm itself and that the variations in conductivity are due to changes in the living protoplasm and not to changes in the intercellular substance (unless cases should be found where ma-

terial killed as described above shows variations in conductivity similar to those found in living tissue).[1]

If, in spite of this demonstration, the question be raised whether determinations of conductivity really measure the permeability of the protoplasm, it may be answered that this method gives results of the same character as the method of plasmolysis, which has been generally relied upon for trustworthy information regarding permeability. In addition, the results obtained by the electrical method are in complete agreement with those obtained by a variety of other methods of measuring permeability, such as that of tissue tension, exosmosis, and diffusion through membranes of living tissue.[2]

This general agreement must mean that the electrical method measures permeability just as the other methods do.[3] It is, however, more accurate as well as more convenient. It enables us to make determinations at much shorter intervals than other methods. This not only permits the detection of rapid changes in permeability which would otherwise escape observation, but it enables us to determine with precision the time curve, which is indispensable in the study of dynamics.

[1] It is necessary to make sure that during the experiment the intercellular spaces should not change in size (as by the contraction of the protoplasm) or in content (as by the displacement of gas by liquid). This source of error was carefully guarded against in the experiments here described.

[2] Cf. Brooks, S. C., *Proc. Nat. Acad. Sc.*, 1916, ii, 569.

[3] The method may be applied to intact organisms as well as to pieces of tissue. Experiments with intact fronds of *Laminaria* give the same results as those made with pieces cut from the fronds.

NOTE ON THE EFFECT OF DIFFUSION UPON THE CONDUCTIVITY OF LIVING TISSUE.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, October 17, 1918.)

The writer has shown that electrolytes may be divided into two classes with respect to their effect on the electrical conductivity of living tissues: (1) those which produce a rise in resistance followed by a fall, and (2) those which produce only a decrease in resistance. Salts with bivalent and trivalent cations belong in the first class while those with monovalent cations belong in the second.

Recently some apparent exceptions to this rule have been met with. The behavior of certain substances is anomalous in three respects: (1) Although they are monovalent salts they produce a rise in resistance, (2) the rise is unusually brief in duration, (3) it occurs in dead as well as in living tissue.

As an example we may take an experiment with artificial sea water. This was made by mixing salts in the following molecular proportions: 1,000 NaCl, 78 $MgCl_2$, 38 $MgSO_4$, 22 KCl, 20 $CaCl_2$, and adding sufficient water to make the electrical conductivity of the solution the same as that of sea water. When tissue of *Laminaria* was transferred from this to an artificial sea water (of the same conductivity as sea water) in which RbCl was substituted for NaCl, there was a sharp rise in resistance which disappeared in a few minutes. On transferring to artificial sea water with NaCl in place of RbCl, there was a drop in resistance.

When the tissue was transferred from artificial sea water made with NaCl to one (of the same conductivity as sea water) in which LiCl was substituted for NaCl, there was a drop in resistance; on replacing in artificial sea water made with NaCl there was a rise.

Similar effects were observed on dead tissue, but here, as was to be expected, the changes were not so great.

It is evident that these effects are due to diffusion. On transferring from artificial sea water made with NaCl to one in which RbCl takes the place of NaCl, the molecules of NaCl diffuse out of the tissue more rapidly than the larger molecules of RbCl can diffuse inward. Hence there is a temporary deficiency of salt in the tissue and the resistance accordingly rises. On the other hand the smaller molecules of LiCl diffuse in faster than NaCl can diffuse out, causing a temporary excess of salt, which lowers the resistance.[1]

Any confusion which might be caused by disturbances of this sort may be easily avoided by control experiments made on dead tissue.

[1] If tissue is transferred from sea water to a solution of RbCl (of the same conductivity as sea water) no rise is observed, since the diffusion effect is more than counterbalanced by the increase in permeability caused by pure RbCl.

THE PROTEINS OF THE PEANUT, ARACHIS HYPOGÆA.

III. THE HYDROLYSIS OF ARACHIN.

By CARL O. JOHNS and D. BREESE JONES.

(From the Protein Investigation Laboratory, Bureau of Chemistry, Department of Agriculture, Washington.)

(Received for publication, October 17, 1918.)

The preparation of arachin,[1] the principal protein in the peanut, *Arachis hypogæa*, has been described in the first paper of this series. The procedure used in the hydrolysis of arachin and the isolation of the amino-acids is in general the same as that described in the hydrolysis of kafirin.[2]

It will be noted in the description of the experimental work that trouble was encountered in extracting the esters from the second esterification. This was finally overcome by removing the bases by means of phosphotungstic acid and esterifying again.

Foreman[3] has called attention to the fact that glutaminic acid readily forms pyrrolidone carboxylic acid in dilute acid or alkaline solutions. He has also shown that glutaminic acid can be formed from pyrrolidone carboxylic acid by hydrolyzing with hydrochloric acid. This information was used in recovering glutaminic acid from the distillation residue.

The percentage of the basic amino-acids was determined by Van Slyke's method as well as by the direct method of Kossel and Kutscher. The values obtained for arginine and histidine by both methods agreed closely. The Van Slyke method showed the presence of more lysine than we succeeded in isolating by the direct method. The results of the hydrolysis are given in Table I.

[1] Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916–17, xxviii, 77.
[2] Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1918, xxxvi, 323.
[3] Foreman, F. W., *Biochem. J.*, 1914–15, viii, 481.

TABLE I.

Hydrolysis of Arachin.

	per cent
Glycine	0.00
Alanine	4.11
Valine	1.13
Leucine	3.88
Proline	1.37
Phenylalanine	2.60
Aspartic acid	5.25
Glutaminic acid	16.69
Serine	—
Oxyproline	—
Tyrosine	5.50*
Cystine	0.85†
Arginine	13.51†
Histidine	1.88†
Lysine	4.98†
Tryptophane	Present.
Ammonia	2.03
Total	63.78

* By the colorimetric method of Folin and Denis.

† Basic amino-acids determined by Van Slyke's method.

EXPERIMENTAL.

500 gm. of arachin, equivalent to 466 gm. of the ash- and moisture-free protein, were hydrolyzed separately in two portions of 200 and 300 gm. each with a mixture of 600 cc. of concentrated hydrochloric acid and 600 cc. of water by boiling in an oil bath for 48 hours. The solution was diluted with water and filtered through norite. In this way the humin was removed and a solution obtained which was nearly colorless. After thoroughly washing the norite with hot water, the washings and the main filtrate were united, the resulting solution was concentrated, and the glutaminic acid separated in the usual way by saturating with hydrochloric acid gas. There were obtained from the two portions 29.00 and 45.16 gm. respectively of glutaminic acid hydrochloride. With the 22.88 gm. subsequently obtained from the esters the total glutaminic acid hydrochloride weighed 97.04 gm., equivalent to 77.76 gm. of glutaminic acid, or 16.69 per cent of the arachin.

The free acid obtained by decomposing the hydrochloride with an equivalent amount of normal potassium hydroxide was analyzed with the following results:

0.2593 gm. substance gave 0.3860 gm. carbon dioxide and 0.1440 gm. water.

	Calculated for $C_5H_9O_4N$:	Found:
C	40.80	40.60
H	6.12	6.21

The filtrates from the glutaminic acid hydrochloride were united, concentrated to a thick sirup, and freed from water by evaporation with alcohol under diminished pressure. The residue of amino-acids was then esterified according to the method of Phelps and Phelps,[4] and the resulting esters were liberated from their hydrochlorides by dissolving in absolute alcohol and adding the calculated amount of sodium necessary to combine with the chlorine, as described in a previous publication.[2] The alcohol was removed from the esters by evaporation under diminished pressure and collected in a receiver containing hydrochloric acid so as to avoid any possible loss of esters by volatilization. The alcohol was saved for further examination. The sirupy residue of esters was then stirred with about a liter of ether, whereupon it soon changed to a stiff, plastic mass. The clear ether extract was removed by decantation. On stirring the semisolid residue with more ether, it gradually became friable and was reduced to a powder. This was extracted with fresh portions of ether several times. The ether extracts were dried over anhydrous sodium sulfate in the usual way. The powdery residue remaining after the extraction with ether was freed from zinc by means of hydrogen sulfide, and subjected to a second esterification. The esterified product on cooling formed a hard, wax-like substance which would not yield to the usual methods of handling the esters at this stage. As this condition of the residue was probably due to the relatively large amount of basic amino-acids present, the esters were hydrolyzed and the bases removed by precipitation with phosphotungstic acid. The solution thus freed from the bases was evaporated to a sirup, and the residual amino-acids were esterified in the usual way. The ether was removed from the esters by distillation at atmospheric pressure. 260 gm. of

[4] Phelps, I. K., and Phelps, M. A., *Am. J. Sc.*, 1907, xxiv, 194.

esters were obtained, 79 gm. of which were obtained from the second esterification.

Alcohol Distilled from the Esters.—The alcohol, which had been distilled from the esters after their liberation from their hydrochlorides with sodium ethylate and which had been acidified with hydrochloric acid, was evaporated to dryness and the residue boiled with water to hydrolyze the esters. After removing the chlorine with silver sulfate there were obtained 8.42 gm. of amino-acids which were added to a similar fraction obtained from the ether, which was distilled from the esters as described below.

Ether Distilled from the Esters.—The ether which was distilled from the esters was acidified with an absolute alcohol solution of hydrochloric acid and allowed to stand at 0° for about a week. There was no separation of glycine ester hydrochloride. The ether was distilled off and the residue boiled with water for 7 hours to hydrolyze the esters. After removing the chlorine with silver sulfate, the solution was evaporated to dryness. To this residue were added the amino-acids obtained from the alcohol distilled from the esters as described above. The united fractions of amino-acids were boiled with absolute alcohol to extract the proline. This alcoholic extract of proline was added to the proline solution obtained from the distilled esters. There were thus obtained about 17 gm. of amino-acids insoluble in alcohol. From this mixture were isolated by fractional crystallization 1.22 gm. of leucine, 9.90 gm. of alanine, and 5.88 gm. of a mixture containing alanine, valine, and leucine. This mixture was added to a similar one obtained from Fractions I and II of the distilled esters.

Analysis showed the leucine to have the following composition.

0.1634 gm. substance gave 0.3282 gm. carbon dioxide and 0.1451 gm. water.

	Calculated for $C_6H_{13}O_2N$:	Found:
C	54.96	54.78
H	9.99	9.94

The alanine had the following composition.

0.2072 gm. substance gave 0.3090 gm. carbon dioxide and 0.1469 gm. water.

	Calculated for $C_3H_7O_2N$:	Found:
C	40.41	40.67
H	7.92	7.93

The esters remaining after the removal of the ether were distilled in the usual way with the following result.

Fraction.	Temperature of the bath up to	Pressure.	Weight.
	°C.	mm.	gm.
I	100	10.0	17.5
II	108	0.8	36.5
III	135	1.0	33.5
Distillation residue			146.0

Fraction I.—This fraction was hydrolyzed with boiling water, the solution of amino-acids evaporated to dryness under reduced pressure, and the proline extracted with boiling absolute alcohol.

By systematic fractionation of the free amino-acids from water, and from water and alcohol, there were obtained from this fraction 0.71 gm. of leucine, 7.51 gm. of alanine, and 2.05 gm. of a mixture consisting chiefly of valine and leucine. This mixture was united with other similar mixtures obtained during the course of this analysis (see below). The presence of glycine could not be established. The leucine gave the following analysis:

0.1329 gm. substance gave 0.2680 gm. carbon dioxide and 0.1198 gm. water.

	Calculated for $C_6H_{13}O_2N$:	Found:
C	54.96	55.00
H	9.99	10.09

Analysis showed the alanine to have the following composition.

0.1445 gm. substance gave 0.2130 gm. carbon dioxide and 0.1050 gm. water.

	Calculated for $C_3H_7O_2N$:	Found:
C	40.41	40.20
H	7.92	8.13

Fraction II.—The esters of this fraction were hydrolyzed by boiling with water until the alkaline reaction to litmus had disappeared. After removing the proline by extraction with absolute alcohol, a residue of amino-acids insoluble in absolute alcohol was obtained consisting of leucine and valine, from which by fractional crystallization 11.11 gm. of leucine were isolated. The

remainder formed an inseparable mixture of leucine and valine. To this mixture was added the fraction consisting of alanine, valine, and leucine obtained from the ether which was distilled from the esters, and also the valine-leucine mixture obtained from the distilled esters of Fraction I. These united portions were then subjected to the lead salt method of Van Slyke and Levene[5] for the separation of leucine and valine. There were thus isolated in the form of the lead salt 5.03 gm. of leucine. The lead leucine gave the following results on analysis.

0.2719 gm. substance gave 0.1767 gm. lead sulfate.

	Calculated for $Pb(C_6H_{12}O_2N_2)_2$:	Found:
Pb	44.29	44.38

The filtrate from the lead leucine, after having been freed from lead with hydrogen sulfide, yielded by fractional crystallization 5.27 gm. of valine and 1.74 gm. of alanine. The valine crystallized in the characteristic lustrous plates, which by analysis were shown to have the following composition.

0.2678 gm. substance gave 0.5067 gm. carbon dioxide and 0.2282 gm. water.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.28	51.60
H	9.47	9.53

The alanine was analyzed with the following results.

0.1714 gm. substance gave 0.2547 gm. carbon dioxide and 0.1240 gm. water.

	Calculated for $C_3H_7O_2N$:	Found:
C	40.41	40.53
H	7.92	8.08

All of the alcoholic extracts of proline were united. After filtering off a small amount of a substance which had separated on long standing, the solution was evaporated under reduced pressure to dryness. The residue was completely soluble in cold absolute alcohol. It was again taken down to dryness and the residue dissolved in water and made up to 500 cc. The total ni-

[5] Van Slyke, D. D., and Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1908–09, vi, 54.

trogen in this solution was 1.0687 gm. and the amino nitrogen 0.2885 gm. The difference, 0.7802 gm., is equivalent to 6.41 gm. of proline.

Fraction III.—The phenylalanine ester of this fraction was removed in the usual way by extraction with ether. It yielded 5.18 gm. of phenylalanine in the form of the hydrochloride. The free phenylalanine gave the following analysis.

0.2032 gm. substance gave 0.4862 gm. carbon dioxide and 0.1219 gm. water.

	Calculated for $C_9H_{11}O_2N$:	Found:
C	65.45	65.26
H	6.66	6.71

There were further obtained from this fraction 9.54 gm. of aspartic acid as the barium salt, and 2.47 gm. as the copper salt. The free aspartic acid, obtained by decomposing the barium salt with sulfuric acid, reddened but did not decompose at 300°C.

0.2065 gm. substance gave 0.2731 gm. carbon dioxide and 0.0978 gm. water.

	Calculated for $C_4H_7O_4N$:	Found:
C	36.09	36.07
H	5.26	5.30

The copper aspartate when recrystallized from water separated in the characteristic sheaves of needles.

0.1729 gm. air-dried substance gave 0.0501 gm. copper oxide.

	Calculated for $C_4H_5O_4N\ Cu.4\frac{1}{2}\ H_2O$:	Found:
Cu	23.07	23.15

Distillation Residue.—The undistilled portion of the esters was shaken with a mixture of ether and water to remove the phenylalanine. On standing there separated from the ether solution about 3.5 gm. of diketopiperazines which were filtered off, the ether being allowed to evaporate spontaneously. The residual esters were hydrolyzed by heating on a steam bath with concentrated hydrochloric acid. About 2 gm. of a black tarry substance separated which was removed by extraction with ether. The acid solution, after having been decolorized with norite, was concen-

trated and yielded 5.40 gm. of phenylalanine hydrochloride. The free phenylalanine, obtained by decomposing the hydrochloride with ammonia, gave the following results when analyzed.

0.1576 gm. substance gave 0.3766 gm. carbon dioxide and 0.0957 gm. water.

	Calculated for $C_9H_{11}O_2N$:	Found:
C	65.45	65.17
H	6.66	6.79

The filtrate from the phenylalanine hydrochloride after having been freed from chlorine and the solution boiled with copper carbonate, yielded 0.66 gm. of copper aspartate which crystallized from water in the characteristic sheaf-like crystals.

There were further obtained from the filtrate from the copper aspartate, after having removed the copper with hydrogen sulfide, 0.3 gm. of tyrosine and 2.5 gm. of phenylalanine.

The aqueous solution remaining after shaking the distillation residue with ether and water was boiled with barium hydroxide to hydrolyze the esters. The barium was removed quantitatively with sulfuric acid and the solution concentrated. After filtering off 3.98 gm. of tyrosine which had separated, the solution was saturated with hydrochloric acid gas, but on standing for several days at 0° no glutaminic acid hydrochloride separated. The hydrochloric acid was removed by evaporation under reduced pressure and finally with silver sulfate, and the solution boiled with copper carbonate. On standing there crystallized 8.18 gm. of copper aspartate.

0.2084 gm. substance gave 0.0609 gm. copper oxide.

	Calculated for $C_4H_5O_4N\,Cu.4\frac{1}{2}\,H_2O$:	Found:
Cu	23.07	23.35

The filtrate from the copper aspartate, after removing the copper with hydrogen sulfide, gave a large precipitate on addition of an excess of phosphotungstic acid. After the solution was freed from the basic amino-acids in this way, it yielded 2.0 gm. of tyrosine, 3.35 gm. of glutaminic acid hydrochloride, 3.36 gm. of copper aspartate, and a considerable amount of a clear, yellow sirup from which on long standing nothing separated. It seemed very probable that considerable of the glutaminic acid originally

present in the distillation residue might have been converted into pyrrolidone carboxylic acid, by having been subjected to the various steps in the foregoing analysis. Foreman[3] has shown that the glutaminic acid can be almost completely recovered by hydrolyzing the pyrrolidone carboxylic acid by boiling with 20 to 25 per cent hydrochloric acid. The above mentioned sirup was accordingly boiled with 25 per cent hydrochloric acid for about 19 hours. The solution was then concentrated to about 75 cc., saturated with hydrochloric acid gas, and allowed to stand for a few days in a refrigerator. A large yield of crystals separated. There were thus obtained 19.53 gm. of pure glutaminic acid hydrochloride which melted with decomposition at 197°C. The free glutaminic acid, obtained by decomposing the hydrochloride with an equivalent amount of normal potassium hydroxide, gave the following results on analysis:

0.2758 gm. substance gave 0.4117 gm. carbon dioxide and 0.1556 gm. water.

	Calculated for $C_5H_9O_4N$:	Found:
C	40.81	40.71
H	6.12	6.31

Tyrosine.—A quantity of arachin equivalent to 1 gm. of the ash- and moisture-free protein was hydrolyzed by boiling with 25 cc. of 20 per cent hydrochloric acid for 11½ hours. The solution was decolorized with norite, made up to 100 cc., and the tyrosine determined colorimetrically according to the method of Folin and Denis.[6,7] The tyrosine found was equivalent to 5.50 per cent of the arachin.

Aspartic Acid.—50 gm. of arachin, equivalent to 46.62 gm. of ash- and moisture-free protein were hydrolyzed by boiling with 200 cc. of 20 per cent hydrochloric acid for 40 hours. The aspartic acid, together with some glutaminic acid, was separated from the other amino-acids by precipitation of their calcium salts according to the method of Foreman.[8] There were finally isolated in pure condition 5.42 gm. of copper aspartate. This is equivalent to 2.61 gm. of aspartic acid or 5.61 per cent of the arachin. The

[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 245.
[7] Johns and Jones, *J. Biol. Chem.*, 1918, xxxvi, 319.
[8] Foreman, *Biochem. J.*, 1914–15, viii, 463.

copper aspartate crystallized from water in the characteristic sheaves, and gave the following results on analysis:

0.2061 gm. substance gave 0.0594 gm. copper oxide.

	Calculated for $C_4H_5O_4N\ Cu.\ 4\frac{1}{2}\ H_2O$:	Found:
Cu	23.07	23.03

The total aspartic acid isolated from the distilled esters was 3.84 per cent, which is 1.77 per cent less than that obtained by the above method.

Basic Amino-Acid.—The bases had been previously determined by Van Slyke's[9] method. The bases were also determined by the direct method of Kossel and Kutscher. This determination gave 12.54 per cent of arginine, 2.13 per cent of histidine, and 1.72 per cent of lysine.

The values obtained for arginine and histidine agree well with those found by Van Slyke's method, while the value found for lysine is much lower.

The lysine was determined by weighing pure lysine picrate. A sirupy residue was left from which more lysine picrate could not be crystallized. This accounts in part for the discrepancy between the results obtained by the different methods.

[9] Johns and Jones, *J. Biol. Chem.*, 1917, xxx, 33.

THE INFLUENCE OF PROTEIN FEEDING ON THE CONCENTRATION OF AMINO-ACIDS AND THEIR NITROGENOUS METABOLITES IN THE TISSUES.

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In the last few years much information has been secured in regard to the metabolism of the proteins, due largely to the elaboration of accurate methods for the determination of amino-acids and their nitrogenous metabolites. As the result of many excellent and highly significant experimental researches, at least the general features of the method by which the mammalian organism utilizes the energy content of protein material have been revealed. The true significance of the amino-acids in relation to protein utilization and protein requirements is now known, and consequently the purpose of the extensive hydrolytic cleavage of proteins in the gastrointestinal tract is no longer ambiguous. There is now no reason to believe that the amino-acids undergo any appreciable change during their passage through the absorptive membranes of the intestines into the blood and lymph, and their ready access in greater or less amounts to all the tissues of the body cannot be doubted. Their subsequent catabolism is known to yield, on the one hand, a series of nitrogenous substances, whose identities and transformations have been determined experimentally with a large measure of success. On the other hand, the residual non-nitrogenous substances simultaneously produced have in the main eluded direct experimental research *in vivo*, a circumstance the more unfortunate, since they contain practically all of the physiologically available energy of the original amino-acids. The identity and the possible transformations to which these latter substances are subjected have of necessity been investigated by indirect methods. Needless to say, the interpreta-

tion of results secured by such methods is not simple. However, from the sum total of experimental data thus obtained certain inferences may be drawn concerning the general courses of breakdown of the non-nitrogenous residues of the various amino-acids, inferences which may reasonably be assumed to possess a high degree of probability.

There are, naturally, some phases of amino-acid catabolism concerning which the experimental evidence obtained is insufficient or even conflicting. For example, the question of the effect of protein feeding on the concentration of amino-acids in the tissues, and of certain of their nitrogenous metabolites, seems still unsettled. A somewhat detailed and critical review of the experimental evidence on this point is in order before considering the additional data to be reported in this paper.

It has been shown repeatedly by many experimental investigations that the amino nitrogen content of the blood may be doubled or even tripled during the digestion of a heavy protein meal, so that as regards the blood there can be no question that protein feeding increases its amino-acid concentration. As regards the muscle tissue, the evidence is not so clear.

Folin and Denis, in their pioneer investigations on protein metabolism, always obtained an increase in the non-protein nitrogen of muscle following the injection of amino-acids, amino-acid digestion mixtures, or Witte's peptone into a ligated loop of intestine, provided that such materials were readily absorbed. Furthermore, the increases thus obtained could not be accounted for by increases in urea or ammonia, and presumably, therefore, were due to the absorption of amino-acids by the muscle tissue from the blood. However, it cannot, of course, be concluded with certainty from these experiments that during the relatively slow liberation of amino-acids in normal protein digestion the amino-acid content of the muscles would increase, though certainly this evidence establishes a degree of probability to that effect.

The experiments of Van Slyke and Meyer along the same general lines are capable of yielding more definite information, since they determined directly the amino-acid nitrogen of their samples by the now standard nitrous acid method. However, most of their analyses were made on the tissues of dogs after the injection

into the venous blood of amino-acids and protein digestion mixtures. Only a relatively few experiments were performed on dogs during normal protein digestion. The experiments on the injection of amino-acids clearly demonstrated the following:

1. The tissues rapidly absorb amino-acids from the blood when their concentration in that fluid is increased. An equilibrium is reached when the tissues contain roughly about ten times the percentage of amino-acid present in the blood. This process is not a mere diffusion, though the tissues do not appear to be in chemical union with the absorbed amino-acids.

2. Muscle and kidney tissue retain all of the amino-acids thus absorbed for 3 or even 4 hours. Apparently there is a latent period of several hours duration between the taking up of amino-acids by the muscles from the blood and the catabolism of the excess amino-acids. The liver, on the other hand, although capable of absorbing more amino-acids per unit of weight than any other organ or tissue examined, does not retain them for any length of time: in 3 or 4 hours the excess amino-acids had disappeared. Furthermore, there seemed to be no reason for assuming that they had been transferred to any other organ or tissue, and since, simultaneously with their disappearance from the liver, there was a rise in the urea content of the blood, the conclusion was drawn that the liver catabolized amino-acids at a very much greater rate than the muscles, and that at least a part of the nitrogen thus formed is converted into urea.

These are the most important facts and conclusions resulting from the experiments on the intravenous injection of amino-acids into dogs. From experiments on fasting dogs and on dogs digesting a heavy protein meal, two results of outstanding importance were obtained: (1) In dogs which, after 48 hours fasting, were given heavy meals of meat, in all cases the urea content of the blood rose almost immediately after the meat was consumed. Roentgenograms showed, in fact, that the rise in urea was simultaneous with the passage of the first particle of chyme from the stomach to the duodenum. (2) A comparison of the tissues of fasting dogs with those of dogs killed a few hours after consuming fresh meat showed that neither the livers nor other tissues of the fed animals contained a definitely greater store of amino-acids than did the tissues of the fasting animals.

From these somewhat remarkable results it was concluded that, unreasonable as it may appear, the organism does not wait until it has absorbed sufficient protein digestion products to meet its immediate requirements and thereafter begin to turn the surplus into urea. The very beginning of absorption stimulates the urea-forming function into activity, this initial urea formation being attributed to the liver. It was also concluded that the concentration of amino-acids in the body is so well regulated, presumably by the liver, that no great fluctuations ordinarily occur, even after heavy consumption of protein. "The digesting dogs must have either destroyed or condensed into protein practically all the amino-acids which they absorbed, and have done so at a rate which was nearly parallel with that of absorption."[1]

The above interpretation of the two experimental results just stated seems open to question. In the first place the formation of urea by the liver from ammonium salts arising from the hydrolysis of protein in the stomach and intestine is not considered. The increase observed in the concentration of urea in the blood cannot with certainty, therefore, be ascribed to the deamination of amino-acids, and such an interpretation may even be considered improbable in view of the close coincidence observed between the appearance of surplus urea in the blood and the first passage of chyme from the stomach to the duodenum. In all probability no amino-acids are liberated during the gastric digestion of proteins, at least in any but negligible quantities, while ammonia is undoubtedly one of the products of peptic digestion and is produced in considerable quantities from most proteins. Hence any urea formed by the liver immediately after the entrance of chyme into the duodenum more probably originated from ammonium salts than from amino-acids. The ability of the liver to convert rapidly ammonium salts of organic acids into urea is well recognized, while its ability rapidly to deaminize amino-acids and convert the resulting ammonia to urea is not so well established, and indeed has been denied by some investigators.

Hence the rate of increase of urea in the blood during protein digestion can give no sure clew to the rate of amino-acid catabo-

[1] For a very complete discussion of Van Slyke's experimental data and the conclusions deduced therefrom, see Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

lism, nor to the time at which this catabolism is initiated. The liver is probably competent to keep the concentration of ammonia in the systemic blood and in the other tissues within very narrow limits under normal conditions of metabolism, by conversion of ammonium salts into urea; that it is competent to do likewise with the concentration of amino-acids is less certain. In fact, if the liver possessed such a capacity, many phases of protein utilization, especially in the growing animal, would be difficult to understand. It may be mentioned, also, that there exists an unexplained contradiction between the results of Van Slyke, which indicate the formation of urea almost immediately after the ingestion of protein, and those of Folin and Denis, which indicate a lag of ½ to 1 hour between the time of injection of amino-acid mixtures into a ligated loop of intestine and an increase in the concentration of urea in the blood.

As regards the second result of the experiments on fed and fasting dogs, which indicated no essential differences in the concentration of amino-acids in the tissues of fasting dogs and of dogs killed at the height of protein digestion, the published data on this point are too few to be particularly convincing.[2] Analyses have been reported by Van Slyke and Meyer of the amino-acid content of the tissues of seven dogs, one killed 5 hours after a meal of 1 pound of fresh meat, and the others killed after fasting 18 hours, 20 hours, and 2, 4, 6, and 12 days. It will be noticed that the comparison must be made between one fed dog, and six dogs fasted for different intervals, all of which, however, were killed in the "postabsorptive period." The data for the fasted dogs are extremely variable among themselves and for no single tissue is there a consistent progressive increase or decrease in amino-acid content with the length of the fasting period. It is true that the data for the single fed dog are not to be distinguished from those for the other animals, but it seems obvious that the data are statistically insufficient to justify drawing positive conclusions. If animals killed 5 hours after a meat meal exhibit as great a variability in the amino-acid content of their tissues as the six fasted dogs, it is quite possible that the one fed dog actually analyzed may have been exceptional in this respect, a surmise some-

[2] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913–14, xvi, 231.

what fortified by the fact that the amino-acid content of the blood of the fed dog was no higher than that of the dog fasted for 18 hours and only slightly higher than that of the dog fasted for 4 days. This relation is quite different from that reported in an earlier paper by the same investigators,[3] in which it is shown that the amino-acid content of the blood of two dogs killed 5 hours after a meal of 1 kilo of fresh beef is about double that of the same dogs before the meal and following a 24 hour fast.

Passing from the data to the interpretation, *i.e.* that digesting dogs must destroy or condense into protein all amino-acids, which they absorb, at a rate parallel to that of absorption, the objection may be raised that such an interpretation is not consistent with the numerous results obtained on dogs following the injection of amino-acids intravenously. These experiments, as mentioned above, showed clearly that the muscles and kidneys retain in undiminished amount the excess amino-acids absorbed from the blood, for as long as 4 hours. No reason is apparent why, in the one case, amino-acid catabolism should be so rapid in the muscles, while, in the other case, it is so long delayed. On the basis of the results obtained in the injection experiments, and of the demonstrated fact that during protein digestion the amino-acid content of the systemic blood is notably increased, one should expect that the amino-acid concentration in the muscles would increase also to an extent corresponding to the amino-acidemia and its duration, and that this increased concentration would persist for several hours. As a matter of fact, Folin and Denis obtained results that may reasonably be interpreted as indicating an increased amino-acid concentration in the muscles following the injection of amino-acid mixtures into the intestine.

In order to understand the extent to which protein nutriment is accessible to the tissues of the body, and the function of the liver in regulating the amino-acid concentration of the tissues, additional information is needed. If the deaminizing function of the liver is as vigorous as Van Slyke assumes, no appreciable change in the amino-acid concentration of the tissues would occur during protein digestion, and as a consequence only a negligible portion

[3] Van Slyke and Meyer, *J. Biol. Chem.*, 1912, xii, 408.

of the amino-acids coming to the liver through the portal vein could be assumed to reach the other tissues of the body. Under these conditions, the growing animal, the animal recuperating from disease or inanition, and the pregnant or lactating female, would be placed under a great handicap in covering their protein requirement, unless a further assumption can be made that under these conditions the deaminizing function of the liver is in some way depressed to allow the tissues to obtain abundant protein nutriment. On the other hand, if the function of the liver is simply to prevent the amino-acid concentration of the blood and tissues from increasing to such an extent as to produce injurious results, that is, if the deaminizing function of the liver operated at full capacity only under the stimulus of an excessive amino-acidemia, then it would be expected that the tissues could at all times secure sufficient protein nutriment without being overloaded and injured, as could be demonstrated experimentally by the determination of moderate increases in the amino-acid concentration of the tissues during protein digestion.

The main purpose of the experiments to be reported in this paper was to determine whether or not an increase in the amino-acid concentration of the tissues actually occurred during protein digestion, and, second, to obtain information if possible, as to the rate of amino-acid catabolism, by investigating the changes in concentration in the tissues of the nitrogenous metabolites of the amino-acids, ammonia, and urea. The investigation was undertaken with the conviction that the accumulation of a considerable amount of data is requisite before satisfactory conclusions can be drawn; the problem is essentially a statistical one. As an additional illustration of the difficulties that may be encountered when the data are too few and too variable to warrant interpretation, reference may be made to an investigation by Miss Wishart, working in Lusk's laboratory, to determine the influence of meat ingestion on the amino-acid content of blood and muscle.[4] The muscle tissue of three fasting dogs was analyzed for total non-protein nitrogen and for urea. The results for non-protein nitrogen other than urea, presumably largely amino-acid nitrogen, were 113, 81, and 145 mg. per 100 gm. of tissue. For two dogs killed 3 hours after the ingestion of 1 kilo of meat, the correspond-

[4] Wishart, M. B., *J. Biol. Chem.*, 1915, xx, 535.

ing values were 115 and 132 mg., and for two dogs killed 5 hours after the meat meal, the values were 101 and 117 mg. While the conclusion drawn was that no positive increase in the amino-acid content of muscle occurs during protein digestion, the disparity existing among the figures for the fasting dogs undoubtedly introduces a considerable element of doubt as to the correctness of the conclusion. However, the work of Wishart, and of Van Slyke and Meyer has induced Lusk to change his theory as to the cause of the specific dynamic effect of the amino-acids. Convinced that no appreciable change in concentration of amino-acids in the tissues occurs during digestion, he now concludes that the amino-acids themselves cannot be the cause of the increased heat production, but that the first products of amino-acid breakdown, keto- or hydroxyl-fatty acids, are probably the cell stimulants.

Methods.

Albino rats were used in this investigation, since, with such small animals, the analysis of the entire carcass presents no particular difficulties. The plan of the experiment was to make analyses of animals killed after a fast of 24 to 48 hours, and, for comparison, of other animals killed at varying periods after the ingestion of a high protein meal. The ration fed to the latter animals consisted of casein (Eimer and Amend) 60 per cent, skim milk powder (Merrell-Soule) 10 per cent, and butter fat 30 per cent. On analysis the ration was found to contain 7.75 per cent of nitrogen. Considering the ingredients used, this nitrogen must have been practically all protein nitrogen.

The animals were all killed with ether, and opened immediately. The gastrointestinal tract was removed from cardia to rectum and thoroughly washed out with physiological salt solution. For most of the animals the liver, and occasionally the kidneys, was removed for separate analysis, together with a sample of muscle tissue weighing generally from 8 to 10 gm., taken from the hind legs which were practically stripped of muscle to provide the sample. For all rats analyzed, the bladder was empty of urine. The carcass and intestinal tract were analyzed together, after passage through a meat grinder. All samples were extracted with boiling ammonia-free water, acidified with acetic acid, according to the recommendation of Van Slyke.[5]

[5] Van Slyke, *J. Biol. Chem.*, 1913–14, xvi, 187.

Each sample was extracted eight times with approximately five times its weight of water. The carcass and intestines were analyzed for ammonia, amino nitrogen, and urea; the separate tissues, for ammonia and urea only. In the former case, ammonia was determined both by the distillation method of Van Slyke[5] and by simple aeration after the addition of potassium carbonate, simultaneously with the urea determination. For the liver, muscle, and kidney samples, only the distillation method was used, urea being determined in the extract freed of ammonia by that means. Amino nitrogen was determined by the nitrous acid method of Van Slyke,[5] always on ammonia-free material, and urea by the method of Van Slyke and Cullen,[6] using a vacuum pump and continuing the aeration for 1 hour. By repeated test, 1 hour was found to be more than sufficient to draw through the solutions 100 liters of air. For both the ammonia and urea determinations approximately 0.02 N sulfuric acid was used, back titrations being made with alkali of equal strength.

At first the amount of ration eaten by each of the fed rats previous to analysis was accurately weighed, but later no record was kept of this, since it was evident that the analytical results obtained bore no relation to the amount of food eaten, possibly because in all cases enough food was taken to keep the stomach well filled up to the time of killing. There seems to be no reason for believing that the amount of food taken into the stomach during a meal, bears any relation to the rate of passage of chyme into the duodenum, except perhaps in cases involving extreme gorging of the stomach.

All the above analyses were run on protein-free solutions. At first, the attempt was made to precipitate the non-coagulable proteins by *m*-phosphoric acid, but difficulties in filtration finally led to the use of five volumes of absolute alcohol for that purpose.[5] Tests on one rat extract indicated that the latter reagent gave not only a more easily filterable precipitate, but also a more complete precipitation. Thus, for one of the fasted rats, the nitrogen not precipitated by *m*-phosphoric acid amounted to 2.072 gm., while that not precipitated by alcohol was only 1.349 gm. At the same

[6] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211; 1916, xxiv, 117.

time, the amino nitrogen in the two cases was practically identical; *i.e.*, 0.247 gm. from the *m*-phosphoric acid precipitation and 0.239 gm. from the alcohol precipitation. In another test on a fed rat, the total non-protein nitrogen by the *m*-phosphoric acid precipitation was 0.551 gm., and by the alcohol precipitation, 0.384 gm. The amino nitrogen in the former case was 102 mg., and in the latter case only 88 mg., an appreciable difference.

Results.

The experimental data obtained on the main portions of the rat carcasses, in some cases exclusive of the special tissue samples, are given in Table I, expressed in mg. of nitrogen per 100 gm. of rat. The ammonia values thus expressed are fairly constant and indicate no clear distinction between fed and unfed animals. The urea values are more variable, and indicate clearly, as would be expected, that in the fed animals the concentration of urea in the tissues is distinctly greater than in the fasted animals. It is a fair conclusion, therefore, that an active amino-acid catabolism was occurring in those animals killed a few hours after feeding.

The amino nitrogen figures show a noticeable tendency to decrease from the earlier to the later experiments. This relation is especially well shown in the case of the fasting animals. For the ten animals killed in the postabsorptive period, there is a nearly continuous decrease from the first to the last. For the thirteen animals killed during active protein digestion, the decrease is marked from November to March, after which the values obtained are quite uniform. Another significant fact shown by Table I is that for rats killed in November there is a rather clear distinction between fed and unfed animals, the amino-acid concentration of the fed animals being uniformly higher than that for the unfed. For rats killed from March to June the difference is not so clear. While the fed rats still appear to have a somewhat higher amino nitrogen value, the average difference is within 10 mg. per 100 gm. of tissue. It may be noted here that the change from *m*-phosphoric acid to alcohol as a protein precipitant was made in January. Therefore, the high amino nitrogen values

obtained in November may have been due in part to this change in technique. It could not, however, account for the differences observed between the fed and fasted animals at that time.

TABLE I.

Non-Protein Constituents of the Rat Carcasses. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.

Date killed.	Fasted rats.					Fed rats.					
	No. of rat.	Body wt.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.	No. of rat.	Body wt. (unfed).	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.	Time since last meal.
1917		*gm.*	*mg.*	*mg.*	*mg.*		*gm.*	*mg.*	*mg.*	*mg.*	*hrs.*
Nov. 9	1	120.0	16	—	136	2	134.4	11	—	152	5
" 16	3	93.6	19	31	95	4	92.2	12	61	141	5
" 23	5	141.5	12	—	84	6	123.4	10	53	102	5
1918											
Jan. 15	7	239.6	12	21	102		—	—	—	—	—
Feb. 4*	8	211.5	9	18	46†						
" 26	9	188.5	10†	28†	60†		—	—	—	—	—
Mar. 14	10	165.9	15	23	63†	11	168.2	14	30	60†	5
Apr. 11		—	—	—	—	12	154.3	12	35	54†	3
" 12	13	132.2	13	17	52†		—	—	—	—	—
" 16		—	—	—	—	14	171.5	10	21	53†	3
May 3	15	143.0	14	32	53†	16	135.7	12†	40†	63†	3
" 9		—	—	—	—	17	127.0	14†	25†	53†	1
" 10	18	172.0	12†	14†	46†	19	154.0	13†	24†	50†	7
June 10	20	138.0	—	9	44†	21	183.0	—	43	58†	3
" 11		—	—	—	—	22	190.5	—	35	56†	5
" 26		—	—	—	—	23	187.3	10	23	63†	3
" 26		—	—	—	—	24	142.0	12	33	59†	7

* This rat was fed 5 hours before killing and ate the ration voraciously. Immediately after consuming an unusual quantity, however, it was evidently in great discomfort, experiencing difficulty in breathing. Upon opening the animal it was seen that the stomach was distended to capacity with food, and that the esophagus, as far up as the pharynx, was also distended with food. No evidence of the passage of food into the intestines was noted, so that for this reason as well as the low urea value subsequently determined, the animal should probably be considered as a fasting subject. Evidently the dyspnea due to the packed esophagus had inhibited all the digestive activities, muscular and chemical.

† These results were obtained by analysis of the carcass minus the liver, the muscle sample, generally 8 to 12 gm., and occasionally the kidneys.

The first possibility that presented itself as to the cause for the progressive changes observed in the amino nitrogen figures was that the age of the animal might be a determining factor in the amino-acid concentration of its tissues as well as the reaction to a protein meal. The rats used in the experiments reported above were obtained from a Massachusetts dealer and were a fairly uniform lot as regards size and apparently age also, being less than half grown when received in October. It seemed possible, therefore, that increasing maturity during the course of the investigation may have been the cause of the progressive change in the amino nitrogen data. To test this possibility, five young rats of the same litter, weighing from 50 to 60 gm., were killed and the entire carcasses analyzed as usual for ammonia, urea, and amino

TABLE II.

Non-Protein Constituents of a Litter of Young Rats. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.

Sex.	Weight, unfed.	Time since feeding.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.
	gm.	*hrs.*	*mg.*	*mg.*	*mg.*
Female	50.5	24+	16	22	80
Male	56.8	24+	15	24	80
Female	50.8	3	20	37	85
Male	60.5	3.5	21	41	102
Female	51.7	5.5	—	38	108

nitrogen. While the age of these animals had not been recorded, since at the time of their birth it was not the intention to use them in this work, they were a well nourished lot and probably of normal size for their age. The animals were killed on June 27, two of them after fasting for 24 hours, two of them from 3 to 3.5 hours after feeding the experimental ration, and one of them 5.5 hours after feeding. The results are given in Table II.

These figures reveal higher values in the young fasting animal for ammonia and amino nitrogen than in more mature fasting animals. In particular there is a distinct increase in all three forms of nitrogen for the fed as compared with the fasting animals. The value of 85 mg. of amino nitrogen for the rat killed 3 hours after feeding is possibly too low, since in the concentration

in vacuo of the solution for this determination, evaporation accidentally proceeded to dryness and the temperature of the bath rose to over 90°C. As Van Slyke[5] has shown, such an accident may lead to a loss of amino nitrogen. We may conclude therefore, that for these young rats the ingestion of a protein meal increases distinctly the concentration of amino-acids and of their nitrogenous metabolites in the tissues.

To confirm this point further, another litter of four rats, somewhat older than the preceding, was killed on July 18 and analyzed. Two of the rats had fasted 24 hours or more, while the other two had been given the experimental ration about 5 hours before. The results are shown in Table III.

TABLE III.

Non-Protein Constituents of a Second Litter of Young Rats. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.

Sex.	Weight, unfed.	Time since feeding.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.
	gm.	*hrs.*	*mg.*	*mg.*	*mg.*
Male	95.0	24+	16	28	61
Female	89.0	24+	17	19	61
Male	76.2	5	29	27	70
"	73.5	5.5	—	40	79

These values reveal essentially the same conditions as those obtained with the other litter of young; while the amino nitrogen figures are lower than those given by the others, due probably to the more advanced development of these animals, there is still an appreciable increase in amino nitrogen due to feeding. The high urea value for the first rat is possibly correlated with the fact that the stomach still contained appreciable amounts of food residue, while the cecum was well filled. With all other fasted animals, the stomach was practically empty. The absence of a high amino nitrogen value also in this rat, may be due to the fact that the animal was apparently killed in the last stages of digestion, when the amino-acid catabolism in the muscles had been in active operation for several hours, had disposed of the excess of amino-acids accumulated in the first stages of digestion, and was deaminizing amino-acids at a rate equal to the absorption from the blood.

When the above analyses were completed, there was one more litter of young left for use in this experiment. It was, however, with some hesitation that these rats were taken, since they were very poorly nourished, had rough coats, and one was afflicted with badly infected eyes. They weighed from 17.7 to 24.2 gm., though they were considerably older than their weight would lead one to expect. In fact, at their age of 38 days these rats should have weighed from 50 to 55 gm. Their poor nutritive condition was due probably to two circumstances: first, the mother had been killed for experimental purposes before the young were properly weaned, and, second, the weather for some time had been extremely hot, and the temperature of the rat quarters had very often been over 102° F. However, with the idea of utilizing all available sources of information, the entire litter was killed for analysis on July 29. The entire litter had been fasted for 24 hours, at which time three were fed the experimental ration and subsequently killed at the end of 1.5, 3, and 3.5 hours. The

TABLE IV.

Non-Protein Constituents of a Third Litter of Young Rats. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.

Weight, unfed.	Time since feeding.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.
gm.	*hrs.*	*mg.*	*mg.*	*mg.*
21.0	24+	13	26	71
18.8*	24+	20	67	91
23.8	1.5	16	28	68
24.2	3	14	22	51
17.7†	3.5	26	52	70

* Dead an hour or more before extraction.

† Both eyes badly infected.

two remaining rats were killed last, without being fed; one was in an extremely weak condition, and the second died an hour or so before the extraction with boiling water could be started. The analytical results secured are given in Table IV.

These results are not at all in agreement with the others reported above, since no definite effect of the protein feeding is evident, even in the case of urea. The high urea and amino nitrogen values for the rat that had been dead for some time before analysis was started, indicate that postmortem changes had already set in. In accounting for the lack of agreement between these results and those secured on normal rats, it is possible that the poor nutritive condition of these animals had impaired their digestive and absorptive powers. Certainly their appetites were not normal, since they displayed no eagerness when food was offered to them and ate sparingly of it, quite contrary to our experience with the other rats. The data are presented simply to complete the record of all evidence collected on the points at issue.

The analytical results obtained on the livers, muscle tissue, and kidneys from the adult rats related only to ammonia and urea nitrogen. It was unfortunate that the amino nitrogen was not also determined, but with the methods used, the samples were insufficient for this purpose. The results are shown in Table V.

TABLE V.

Non-Protein Constituents of Rat Tissues. Results Expressed in Mg. of Nitrogen per 100 Gm. of Tissue.

No. of rat.	Digestive condition.	Weights of tissues.			Urea nitrogen per 100 gm. of			Ammonia nitrogen per 100 gm. of		
		Liver.	Muscle sample.	Kidneys.	Liver.	Muscle.	Kidney.	Liver.	Muscle.	Kidney.
		gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
5	Fasting.	6.1	—	—	—	—	—	10	—	—
7	"	8.5	—	—	35	—	—	16	—	—
8	"	8.464	11.920	—	49	11	—	14	9	—
9	"	7.418	11.324	—	21	—	—	—	—	—
10	"	6.163	8.631	—	22	13	—	41	27	—
13	"	4.382	6.996	1.139	4	13	36	29	11	65
15	"	6.956	9.317	1.323	17	13	—	—	—	—
18	"	9.010	9.418	1.761	16	—	—	—	—	—
20	"	5.888	8.309	—	19	11	—	22	15	—
17	1 hr. after meal.	5.979	7.531	1.067	—	—	—	—	—	—
12	3 hrs. " "	6.232	7.735	1.338	22	25	44	33	22	80
14	3 " " "	8.420	10.014	1.501	22	16	116	16	16	132
16	3 " " "	7.723	7.747	1.356	—	—	—	—	—	—
21	3 " " "	7.337	20.794	—	25	18	—	21	9	—
23	3 " " "	8.587	10.545	—	29	23	—	22	16	—
6	5 " " "	5.4	—	—	—	—	—	11	—	—
11	5 " " "	7.361	10.410	—	34	24	—	23	25	—
22	5 " " "	7.493	11.688	—	26	20	—	16	17	—
19	7 " " "	5.612	8.911	1.268	—	—	—	—	—	—
24	7 " " "	6.207	8.597	—	21	18	—	25	19	—

The urea content of the liver is generally greater than that of muscle tissue, a fact confirming the general opinion that the liver is more active in urea formation than is muscle. The liver exceeds the muscles also in its concentration of ammonia, in most cases. Erratic figures for the kidney may be expected from the smallness of the sample and its variable content of urine.

DISCUSSION.

The non-protein constituents of rat tissue are quite comparable in concentration with those of dog tissue, for example, a confirmation of the tacitly accepted opinion that mammalian metabolism is much the same, regardless of the particular species of animal under consideration. However, a fact discovered in this investigation and not noted in other work, so far as the author is aware, is that the ammonia and amino-acid concentration of young animals is greater than that of adult animals. Whether this relation represents simply a different anatomical make-up of the young animal, such as a greater proportion of viscera and a smaller proportion of muscle, than of the adult, or whether, in addition, it is concerned possibly in the more intense metabolism of the young, it is perhaps premature to say. The difference seems to be greater, however, than can be accounted for on anatomical grounds alone. Possibly the more rapid and general synthesis of protein in the tissues of the young animal may require at all times a greater concentration of amino-acids and ammonia upon which to draw, which may be assured by a greater avidity of the tissues for these substances and a less intense catabolism of them than in the adult.

Perhaps the most important and interesting result of the investigation is the evidence secured as to the effect of protein feeding on the amino-acid concentration of the tissues. Here again, the age of the animal seems to be a determining factor. In the vigorously growing animal, during protein digestion there is a distinct increment in the amino-acid content of the tissues, while as the rate of growth decreases this increment becomes smaller, until in the adult it is only of moderate or perhaps even insignificant dimensions. Apparently there is a relation between the amino-acid requirements of the tissues and their accessibility to the end-products of protein digestion. In explaining such a relation several possibilities suggest themselves: (1) The digestive and absorptive powers of the young organism are more vigorous than of the old, so that of a given protein ration, more is digested and absorbed in a given time, producing a higher amino-acidemia and, by this factor alone, a greater increase in the amino-acid concentration of the tissues. (2) The deaminizing function of the

tissues, especially of the liver, in the young animal is less intense than in the old, permitting a greater influx of amino-acids through the portal circulation and a more delayed catabolism in the tissues. (3) The tissues of the young animal possess a greater absorptive power for amino-acids than those of the adult, so that the equilibrium in amino-acid concentration between tissue and blood is considerably more than 10:1.

However the relation between amino-acid concentration of the tissues and the age of the animal may be explained, the fact remains that in the adult animal only an inconsiderable increase in amino-acid concentration during protein digestion has been demonstrated. It is quite possible that the large percentage of fat in the experimental ration impeded the emptying of the stomach to such an extent that the absorption of amino-acids was relatively slow, and that no considerable accumulation of amino-acids occurred in the blood, and consequently none in the tissues. However that may be, the results obtained confirm the viewpoint of Van Slyke for the grown animal. Whether the liver is the prime factor in the regulation of the amino-acid concentration of the tissues seems questionable, since amino-acids undoubtedly accumulate in the systemic blood during normal protein digestion, even when no considerable accumulation can be demonstrated in the tissues. The more plausible explanation seems to be that the tissues catabolize the amino-acids about as rapidly as they are absorbed from the blood.[7]

Some consideration of the significance of these facts on the problem of the cell stimulants concerned in the specific dynamic effects of amino-acids may not be out of place. The failure to demonstrate a considerable increase in the amino-acid concen-

[7] This interpretation, of course, seems inconsistent with the experimental results of Van Slyke and Meyer, indicating that when amino-acids are injected into the circulation, they accumulate to some extent in the muscles and are retained there for several hours unchanged. However, the constant high amino nitrogen figures observed in muscle for 3 or 4 hours after amino-acid injection may not indicate necessarily a total absence of catabolic activity. They might also be interpreted as meaning a slow catabolism in the muscles, compensated for by a replenishing of the store of amino-acids there from the liver and possibly other tissues. While the data obtained offer no particular support for such an interpretation, as Van Slyke and Meyer state, they are nevertheless consistent with it.

tration of the tissues of adult animals during protein digestion at a time when the specific dynamic effect of protein is being exerted does not necessarily rule out the unchanged amino-acids as the actual stimulants. It is quite conceivable that the qualitative make-up of the amino-acid fraction of a tissue bears some relation to its rate of heat production, quite aside from any change in the size of this fraction. In the postabsorptive period it seems reasonable to suppose that the proteins of the tissue and the free amino-acids and peptides held in it have reached, or approximated to some sort of an equilibrium, which is suddenly disturbed at times when there is an influx of amino-acids from the intestines, constituting a mixture quite different from that normal to the tissue. This disturbance in tissue equilibrium may possibly initiate chemical changes leading to an increased tissue metabolism until a readjustment is made as the resultant of catabolic and anabolic transformations, and after several hours an equilibrium is again established. The fact, as Lusk has shown, that the different amino-acids occurring in proteins have very different specific dynamic effects, perhaps lends some support to such theoretical considerations.

The revised conclusion of Lusk that the α-hydroxyl- and α-keto-acids arising in the metabolism of the amino-acids are the cell stimulants rather than the unchanged amino-acids themselves, does not remove all difficulties. In the first place there can be no assumption that these bodies accumulate in the tissues; in the absence of all direct evidence even that they exist in the tissues in any amount than traces. Nor is it plausible that they are formed only during protein digestion. The occurrence of the constant fraction of amino-acids in the tissues, even during prolonged fasting, probably indicates, as Van Slyke concludes, a continual building up and breaking down of protein material, with, therefore, a continual liberation of the α-hydroxyl- and α-keto-acids arising from the oxidative deamination of amino-acids. As far, therefore, as the quantitative occurrence in the tissues of unchanged amino-acids is concerned, on the one hand, and of the products of their deamination, on the other, there is little to choose in deciding which substances are responsible for the specific dynamic effect of protein. Against deciding in favor of the deaminized acids, it may be argued that the trend of

recent investigation has been to bring out the intimate relationship between the intermediary products of the metabolism of the simpler amino-acids and of glucose. For example, the large specific dynamic effect of alanine cannot with certainty be ascribed to the lactic acid supposed to arise from it in metabolism, since it is, perhaps, quite as probable that lactic acid is produced also in the normal metabolism of glucose, a substance possessing only a slight specific dynamic effect. Furthermore, hydroxyl- and keto-acids are produced in the metabolism of the fatty acids, and, while they are structurally different from those arising in the metabolism of amino-acids, it would be surprising if, in the one case, a large heat effect were produced in the tissues, while, in the other case, practically none at all results.

Against deciding upon the unchanged amino-acids as the stimulants to increased cellular metabolism, it has been argued by Lusk that Rubner, and later Hoobler, have shown that amino-acids, absorbed by the tissues and used in the formation of new tissue, have no specific dynamic effect. However, the work of Howland,[8] in Lusk's laboratory, on the effect of casein on the heat production of a sleeping infant, may be interpreted as indicating a specific dynamic effect of the protein fraction retained for growth. In any case, the point at issue is of such importance and general interest as to require careful confirmation with the best and latest available methods. The peculiar sensitiveness of the infant to protein stimulation as compared with the adult, noticed by Howland, may perhaps be related to the fact established in this investigation that protein feeding in the young organism, as contrasted with the old, leads to a marked increase in the amino-acid concentration of the tissues.

CONCLUSIONS.

1. The concentration of amino-acids, ammonia, and urea in the tissues of rats is comparable to that of the tissues of other mammals thus far investigated.

2. In the young growing rat the concentration of amino-acid and of ammonia in the tissues is considerably higher than in the older animal.

[8] Howland, J., *Z. physiol. Chem.*, 1911, lxxiv, 1.

3. In adult rats, protein feeding has only an inconsiderable effect upon the amino-acid concentration of the tissues, while increasing distinctly the urea content.

4. In young growing rats, on the contrary, protein feeding increases considerably the amino-acid and urea content of the tissues, and, less certainly, the ammonia content.

5. The ammonia and urea content of the livers of rats, both fasting and fed, is in general higher than that of the muscles.

This investigation was undertaken in cooperation with Mr. H. A. Shonle, who, however, withdrew from the University soon after the work was started. Acknowledgment is due to Mr. Shonle for valuable aid in deciding upon methods of procedure, in testing and revising such methods, and in performing some of the analytical work. The assistance of Mr. R. W. Dawley in the remainder of the analytical work is also appreciated. I also wish to acknowledge the interest and encouragement of Dr. H. S. Grindley, in whose laboratory the work was done.

URINARY EXCRETION OF PHOSPHATES IN THE RABBIT.

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The statement is commonly made that the phosphorus in the diet of carnivora is eliminated principally by the kidneys, while, in herbivora, phosphorus excretion takes place almost entirely through the intestine. Bischoff,[1] Müller,[2] Paton, Dunlop, and Aitchison,[3] Bergmann,[4] and others have shown that a large percentage of the phosphorus of the food intake is excreted by dogs in the urine. The urine of herbivora has, on the other hand, been stated by Bertram,[5] Jordan,[6] Tangl,[7] Emery and Kilgore,[8] Paton, Dunlop, and Aitchison,[3] and Bergmann,[4] to be practically free from phosphorus. The types of herbivora used in these studies were the ox, sheep, goat, and horse. The absence of phosphorus from the urine of herbivora is generally attributed to the high content of alkali compounds and calcium salts in the food, an alkaline urine containing carbonates having no solvent power for calcium phosphate. Weiske[9] found that, while on a vegetable diet only a trace of phosphorus was present in the urine of the goat, when fed on a diet of milk, this animal excreted over 20

[1] Bischoff, E., *Z. Biol.*, 1867, iii, 309.

[2] Müller, F., *Z. Biol.*, 1884, xx, 327.

[3] Paton, D. N., Dunlop, J. C., and Aitchison, R. S., *J. Physiol.*, 1899–1900, xxv, 212.

[4] Bergmann, W., *Arch. exp. Path. u. Pharmakol.*, 1901, xlvii, 77.

[5] Bertram, J., *Z. Biol.*, 1878, xiv, 335.

[6] Jordan, W. H., *Ann. Rep. Maine Agric. Exp. Station*, 1885–86, 42.

[7] Tangl, F., cited in *Jahresb. Fortsch. Thierchem.*, 1902, xxxi, 797.

[8] Emery, F. E., and Kilgore, B. W., *North Carolina Agric. Exp. Station, Bull. 118*, 1895, 245.

[9] Weiske-Proskau, H., *Z. Biol.*, 1872, viii, 246.

per cent of the food phosphorus in the urine. Paton, Dunlop, and Aitchison[3] reported that in dogs on a vegetable diet a large proportion of the phosphorus of the food was not excreted in the urine. Gouin and Andouard[10] showed that a calf receiving a diet of milk alone excreted 80 per cent of the food phosphorus in the urine and that phosphorus was deflected from the urine to the feces in proportion to the extent to which vegetable foods were substituted for milk in the diet.

The attention of the authors was attracted to the influence of diet on the urinary phosphorus excretion of rabbits during recent studies on alterations in the output of certain urinary constituents as determined by changes in the character of the diet.[11] It was noted that the proportion of the food phosphorus excreted in the urine varied widely according to the nature of the diet. Upon a diet of corn and oats the urinary output of phosphorus was far in excess of the intake of this element in the food. This excessive output of phosphoric acid in the urine when the animal was placed on a diet which contained a preponderance of acid-forming elements was regarded as a method of regulating acid-base equilibrium in the body of the rabbit. This is in accord with the work of Fitz, Alsberg, and Henderson,[12] who demonstrated by feeding hydrochloric acid to rabbits that the resultant shifting of the salt equilibrium toward the acid side caused an increased elimination of phosphates in the urine. The same effects were observed by the present authors in the studies cited above[11] after giving hydrochloric acid by stomach tube to rabbits.

As a result of these observations the series of experiments to be outlined in this paper was undertaken. The objects were to study further the relative proportions of phosphorus of the food intake excreted in the urine when the diet varied from acid-producing food, such as grain, to base-producing food, like carrots; to note the effect of subcutaneous injections of phosphates on the urinary phosphorus of the rabbit; and to observe the influence

[10] Gouin, A., and Andouard, P., *Soc. aliment. rationn. bétail*, Compt. rend., 1907, 11th cong., 11–28, 127–130.

[11] Underhill, F. P., and Bogert, L. J., *J. Biol. Chem.*, 1916, xxvii, 161.

[12] Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113.

of diets consisting of acid-producing or base-producing foods and of injections of mono-, di-, and tribasic phosphates upon the relative proportion of injected phosphorus excreted in the urine.

Methods.

Rabbits were confined in metabolism cages and the urine was collected in 24 hour periods by expressing it from the bladder by pressure through the abdominal wall. Each animal received either 100 gm. of oats, 500 gm. of carrots, or 50 gm. of oats and 250 gm. of carrots per day. The amounts actually eaten were recorded in the protocols. The phosphates in the urine were determined by titration with uranium acetate, while hydrogen ion concentration was estimated by the Henderson-Palmer method. Subcutaneous injections of solutions of the different sodium salts of phosphoric acid were given in turn at intervals of a few days and repeated with altered dietary conditions. The solutions of sodium phosphates were made up and standardized so that the 30 cc. of injected fluid contained, in each case, 150 mg. of phosphorus. There were no noticeable evil effects of the injections except local irritation caused by the injection of solutions of tribasic sodium phosphate. As there seemed to be a diminished urinary excretion of the injected phosphates after several injections had been given, a further series of experiments was carried out in which the rabbits received successive injections of the same phosphate solution (Na_2HPO_4) while the diet remained constant. For the sake of brevity, specimen protocols only are given from each series of experiments.

Rabbit 7, Male (Black); Weight 2,120 Gm. (Oct. 23).

Date.	Urine volume.	Specific gravity.	pH	Phosphorus.	Notes.
1916	*cc.*			*mg.*	
Oct. 23	77	1.024	8.0	50	50 gm. oats, 228 gm. carrots.
" 24	172	1.013	7.48	79	50 " " 250 " "
" 25	76	1.038	9.27	48	50 " " 250 " "
" 26	96	1.028	8.7	167	50 " " 237 " "
					Injected (subcutaneous) 150 mg. P = 0.68 gm. Na_2HPO_4 in 30 cc. H_2O.
Oct. 27	96	1.038	9.27	69	50 gm. oats, 250 gm. carrots.
" 28	120	1.028	8.7	87	50 " " 250 " "
" 29	130	1.014	8.7	63	50 " " 250 " "
" 30	208	1.017	8.7	145	50 " " 250 " "
" 31	150	1.016	7.48	198	50 " " 250 " "
					Injected 150 mg. P = 0.79 gm. Na_3PO_4 in 30 cc. H_2O.
Nov. 1	190	1.016	8.7	125	50 gm. oats, 250 gm. carrots.
" 2	114	1.017	8.7	100	50 " " 250 " "
" 3	180	1.021	8.7	51	50 " " 250 " "
" 4	164	1.015	8.0	46	50 " " 250 " "
" 5	163	1.017	8.0	86	50 " " 250 " "
" 6	152	1.017	6.9	150	50 " " 250 " "
					Injected 150 mg. P = 0.58 gm. NaH_2PO_4 in 30.7 cc. H_2O.
Nov. 7	152	1.016	7.48	87	50 gm. oats, 250 gm. carrots.
" 8	166	1.017	8.7	87	50 " " 250 " "
" 9	305	1.011	8.0	65	500 " "
" 10	380	1.012	8.7	138	Wt. 2,100 gm. 500 " "
" 11	335	1.010	8.0	97	500 " "
" 12	427	1.013	8.7	114	500 " "
" 13	385	1.011	8.7	107	500 " "
" 14	280	1.015	7.48	117	500 " "
					Injected 150 mg. P = 0.68 gm. Na_2HPO_4 in 30 cc. H_2O.
Nov. 15	460	1.011	8.0	97	500 gm. carrots.
" 16	340	1.011	8.0	66	500 " "
" 17	355	1.011	8.7	79	500 " "
" 18	420	1.011	8.7	81	500 " "
" 19	320	1.010	8.7	101	500 " "
" 20	360	1.013	7.48	135	500 " "
					Injected 150 mg. P = 0.58 gm. NaH_2PO_4 in 30.7 cc. H_2O.

Rabbit 7—Concluded.

Date.	Urine volume.	Specific gravity.	pH	Phosphorus.	Notes.
1916	*cc.*			*mg.*	
Nov. 21	430	1.011	8.7	73	500 gm. carrots.
" 22	290	1.016	8.7	51	500 " "
" 23	383	1.014	8.7	127	500 " "
					Injected 150 mg. P = 0.79 gm. Na_3PO_4 in 30 cc. H_2O.
Nov. 24	350	1.016	8.7	66	500 gm. carrots.
" 25	440	1.014	8.7	61	500 " "
" 26	318	1.017	8.7	61	500 " "
" 27	58	1.034	8.7	72	Wt. 2,060 gm. 100 gm. oats.
" 28	31	1.044	6.9	76	100 gm. oats.
" 29	21	1.060	6.0	72	100 " "
" 30	30	1.052	5.7	104	75 " "
Dec. 1	37	1.041	5.3	149	32 " "
					Injected 150 mg. P = 0.68 gm. Na_2HPO_4 in 30 cc. H_2O.
Dec. 2	29	1.052	5.7	108	48 gm. oats.
" 3	33	1.046	5.3	107	57 " "
" 4	31	1.042	5.3	90	75 " "
" 5	71	1.017	5.3	107	Wt. 2,140 gm. 22 gm. oats.
					Injected 150 mg. P = 0.58 gm. NaH_2PO_4 in 30.7 cc. H_2O.
Dec. 6	30	1.036	5.3	85	31 gm. oats.
" 7	40	1.033	5.3	95	10 " "
" 8	110	1.017	5.3	182	Refused food. Injected 150 mg. P = 0.79 gm. Na_3PO_4 in 30 cc. H_2O.
Dec. 9	40	1.030	5.3	90	Refused food.
" 10	87	1.025	5.3	156	" " Albuminuria.
" 11					Returned to pen, condition fair. Fed carrots, condition good.

Rabbit 12, Female (Brown, Pregnant); Weight 2,840 Gm. (Dec. 11).

Date.	Urine volume.	Specific gravity.	pH	Phosphorus.	Notes.
1916	*cc.*			*mg.*	
Dec. 11					415 gm. carrots.
" 12	255	1.013	8.0	50	352 " "
" 13	158	1.018	8.0	77	345 " "
" 14	305	1.015	8.0	75	500 " "
" 15	110	1.025	7.48	184	230 " "
					Injected 150 mg. P = 0.68 gm. Na_2HPO_4 in 30 cc. H_2O.
Dec. 16	81	1.035	8.7	85	324 gm. carrots.
" 17	234	1.015	8.7	54	403 " "
" 18	90	1.031	7.48	173	228 " "
					Injected 150 mg. P = 0.68 gm. Na_2HPO_4.
Dec. 19	100	1.036	9.27	62	390 gm. carrots.
" 20	196	1.028	9.27	66	362 " "
" 21	150	1.023	9.27	161	370 " "
					Injected 150 mg. P = 0.68 gm. Na_2HPO_4.
Dec. 22	174	1.021	9.27	60	445 gm. carrots.
" 23	234	1.020	9.27	80	457 " "
" 24	222	1.019	9.27	140	474 " "
					Injected 150 mg. P = 0.68 gm. Na_2HPO_4.
Dec. 25	207	1.023	9.27	87	495 gm. carrots.
" 26					Returned to pen, condition good. Wt., 2,880 gm.

DISCUSSION.

Upon inspection of the protocols and tables, several facts of interest are to be noted. First, there is a greater excretion of phosphorus by the urine in the case of the rabbit than in other herbivora previously studied and secondly, this urinary phosphorus may be made to vary largely by changes in the diet. Thus rabbits, even on a diet composed exclusively of carrots, where a strongly alkaline urine is secreted, eliminate about 25 per cent of the food phosphorus by way of the kidneys, while on a diet of oats more than 100 per cent of the food phosphorus is

excreted in the urine. When a mixed diet of oats and carrots is fed, the urine contains about 50 per cent of the phosphorus ingested in the food. The urine of the animals consuming both the carrot and mixed diets is alkaline, but that of the rabbits fed on the grain diet is acid. Moreover, the calcium content of the food is highest in the carrot diet and lowest in the grain diet. Doubtless both the reaction of the urine and the calcium content of the diet are factors in effecting alterations in urinary phosphate excretion by changes in the character of the diet. Difference in utilization of the various diets is another possible factor to be taken into consideration. A summary of these results is found in Table I.

TABLE I.

Effect of Alterations in the Character of the Diet upon the Urinary Excretion of the Ingested Phosphorus.

Rabbit No.	P intake per day.			Average urinary P per day.			P excreted in urine per day.		
	Mixed diet.	Carrot diet.	Grain diet.	Mixed diet.	Carrot diet.	Grain diet.	Mixed diet.	Carrot diet.	Grain diet.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
4	165	240	90	87	59	112	52	24	124
5	165	240	90	50	62	89	30	26	99
6	165	240	90	101	65	110	61	27	122
7	165	240	90	89	63	108	53	26	120
Average.....				82	62	105	49	26	116

In respect to the fate of injected phosphates, there has been a fairly general agreement that such phosphates were excreted by carnivora chiefly in the urine and by herbivora almost entirely in the feces. Falck[13] found that sodium phosphate injected intravenously into dogs was quickly eliminated by the kidneys. The same result was obtained by Bergmann[4] after injecting dogs subcutaneously with sodium phosphate. He reported, however, that the urinary phosphorus of a wether remained unweighable after similar injections of phosphate. Paton, Dunlop, and Aitchison[3] state that, in the goat, none of the phosphoric acid subcutaneously injected as sodium phosphate is excreted in the urine while, in

[13] Falck, C. P., *Virchows Arch. path. Anat.*, 1872, liv, 173.

the dog as well, a large proportion of such injected phosphate is not excreted in the urine. Mendel and Underhill[14] injected both sodium phytate and disodium acid phosphate subcutaneously during a series of balance experiments on a dog with the result that the excess of phosphorus was eliminated almost entirely through the kidneys rather than in the feces. After similar injections in a goat, they found no increase in the phosphates of the urine.

The present series of experiments shows that the rabbit, in contrast to such other herbivora as the goat, excretes an excess of phosphates to a large extent through the kidneys. While there were considerable variations in the percentage of injected phosphates which appeared in the urine, injections of any one of the

TABLE II.

Effect of Successive Injections of Phosphate on the Urinary Excretion of Injected Phosphorus.

Rabbit No.	Diet.	Substance injected.	Injected P excreted in urine. Injection No.			
			1	2	3	4
			per cent	*per cent*	*per cent*	*per cent*
12	Carrot.	Na_2HPO_4	73	78	63	40
14	"	Na_2HPO_4	36	47	30	0
18	Mixed.	Na_2HPO_4	89	80	80	61

three phosphates of sodium invariably caused a distinct rise in urinary phosphates, a rise frequently equivalent to as much as 70 to 100 per cent of the injected phosphorus. So far as we were able to observe, the nature of the phosphate injected or of the diet consumed had no constant effect on the amount of injected phosphorus excreted in the urine. Such observations were complicated by variations in phosphate elimination in individual rabbits and by the effect of successive injections of phosphates. These successive phosphate injections seem either to lessen the ability of the animal to excrete the injected phosphorus through the kidney, to develop an increased excretion of phosphate by the intestine, or to lead to phosphorus storage in the organism. The fact that, after successive subcutaneous injections of sodium

[14] Mendel, L. B., and Underhill, F. P., *Am. J. Physiol.*, 1906–07, xvii, 75.

phosphates, less of the injected phosphorus is excreted in the urine than after the first injection is shown by the experiments on Rabbits 12, 14, and 18, summarized in Table II. Table III shows the percentage of injected phosphorus excreted through the kidneys by various rabbits after initial injections of phosphate, the nature of the diet and of the phosphate injected being altered in different cases.

The effect of subcutaneous injections of phosphates on the hydrogen ion concentration of the urine was not pronounced.

TABLE III.

Urinary Excretion of Injected Phosphorus after Initial Injections of Phosphates with Varying Dietary Conditions.

Rabbit No.	Diet.	Substance injected.	Injected P excreted in urine.	
			per cent	
4	Mixed.	Na_2HPO_4	96	
5	"	Na_2HPO_4	29	
6	"	Na_2HPO_4	80	
7	"	Na_2HPO_4	79	
16	"	Na_2HPO_4	105	
18	"	Na_2HPO_4	89	
19	"	Na_2HPO_4	55	Average 76
12	Carrot.	Na_2HPO_4	73	
13	"	Na_2HPO_4	75	
14	"	Na_2HPO_4	35	Average 61
8	Grain.	Na_2HPO_4	80	
9	"	Na_2HPO_4	50	
10	"	Na_3PO_4	67	
11	"	NaH_2PO_4	73	Average 67

The increased content of the urine in phosphates following such injections was usually accompanied by a slight rise in hydrogen ion concentration but, as might be expected in such a mixture of buffer substances, relatively enormous increases of phosphate were observed with but slight alteration in the reaction of the urine.

SUMMARY.

Rabbits, in contrast to most other herbivora, excrete a considerable amount of phosphates through the kidneys.

The proportion of food phosphorus eliminated in the urine by rabbits may be caused to vary widely by changes in the nature of the diet, being about 25 per cent on a carrot diet, 50 per cent on a mixed diet of carrots and oats, and over 100 per cent on a diet composed exclusively of oats.

Solutions of mono-, di-, and trisodium phosphates injected subcutaneously each caused a considerable increase in urinary phosphates, 70 to 100 per cent of the injected phosphorus having frequently appeared in the urine.

After successive subcutaneous injections of phosphate, less of the injected phosphorus is excreted in the urine than after the initial injection.

The character of the diet and the nature of the phosphate injected had no apparent influence on the percentage of injected phosphorus excreted by the kidneys. It is impossible, however, to draw general conclusions with regard to these factors on the basis of these experiments, due to variations in urinary phosphorus excretion in individual rabbits and to the effect of repeated injections of phosphate solutions on the urinary elimination of phosphates subsequently injected.

Large increases in the phosphate content of the urine were noted, accompanied by only slight increases in hydrogen ion concentration.

STUDIES IN THE METABOLIC CHANGES INDUCED BY THE ADMINISTRATION OF GUANIDINE BASES.

V. THE CHANGE OF PHOSPHATE AND CALCIUM CONTENT IN SERUM IN GUANIDINE TETANY* AND THE RELATION BETWEEN THE CALCIUM CONTENT AND SUGAR IN THE BLOOD.

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In a recent communication (1, 2) it was demonstrated that a marked acidosis appears in the condition of guanidine tetany. This same phenomenon is present in parathyroid tetany and in idiopathic tetany (3) in both of which conditions the same evidences are observed in the urine, such as increased elimination of NH_3, decreased acid excretion, and the lowering of the hydrogen ion concentration. Greenwald (4, 5, 6) found that in parathyroid tetany in dogs the elimination of phosphate was distinctly diminished and that this diminution was accompanied by a marked retention of phosphate in the blood. We suggested that the phosphate retained served to neutralize the acid probably produced by the muscular activity caused by the increased content of guanidine base present when the parathyroid is removed (7, 8, 9).

In connection with the study of the cause of tetany, several investigators estimated the change of salt excretion, especially calcium, in the urine and feces, and the content in the tissues, especially the brain. Among these workers Sabbatani (10), first suggested that the decreased content of calcium in the brain caused the irritability of the nervous tissue, since he had observed that when calcium chloride was applied to the cortical surface the irritability was immediately reduced. With antagonistic agents, such as sodium citrate, the reverse is true. He thought this might be a factor contributing to the cause of convulsive diseases. Following the above suggestion Quest (11) first found that the calcium content of the brains of patients dying in tetany was distinctly diminished when compared to the normal brain of the same age. There was also an increased ratio of nitrogen to calcium. Later workers, including Aschenheim (12) in idiopathic tetany,

* The preliminary work on this subject was reported in the Proceedings of the Society for Experimental Biology and Medicine for May 15, 1918.

and MacCallum and Voegtlin (13), Pexa (14), Aschenheim (12), Weigert (15), in tetania parathyreopriva also state that the content of calcium in the brain is diminished. On the other hand Stoeltzner (16), Leopold and von Reuss (17), and Cohn (18), among others, claim that the calcium content of the brain is approximately normal in idiopathic tetany. Cooke (19) likewise reported that the calcium content of the brain in two dogs in experimental tetany, did not change or was rather slightly higher than in control animals. The magnesium was also practically normal.

Comparing the normal arterial blood in dogs with that after parathyroidectomy, MacCallum and Voegtlin (13), found a marked decrease in calcium and they state that this decrease in the blood and tissues is caused by the increased excretion in the urine and feces. Neurath (20) also demonstrated the diminution of precipitable calcium in the blood.

Beside the work of MacCallum and Voegtlin on the calcium excretion cited above, Cooke (19) claims that the elimination of calcium in the urine is almost unchanged but is increased in the feces. The magnesium excretion is markedly increased in the urine. Recently Kojima (21) stated in his metabolism experiment in rats that after parathyroidectomy there seems to be an increase of calcium in the urine and less retained in the body. Von Cybulski (22) and Schwarz and Bass (23), working with patients suffering from idiopathic tetany in feeding experiments, state that upon the appearance of the tetanic symptoms the calcium retention is lowered. We must also call attention to the work of Iselin (24), Hohlbaum (25), Toyofuku (26), and Morel (27) in which they demonstrated a delayed bone growth, delayed healing of fractures, and insufficient calcification of the teeth in parathyroidectomized rats. The experiments cited above suggest either that the calcium present is changed in some way or that there is an actual change in quantity. Paton and Findlay (28) expressed the opinion that a decreased calcium content of the tissues and an increased elimination of calcium is not a satisfactory explanation although administration of calcium strikingly diminished the electrical excitability and caused a disappearance of the symptoms in parathyroid tetany.

After perusal of the abundant literature concerning this problem, it appears that in parathyroid tetany or in idiopathic tetany the excretion of calcium may be increased in most cases and that at the same time the calcium may be decreased in the blood although all of this evidence is not certain. In the first paper of this series, we mentioned that the true cause of tetany was some toxin, such as guanidine bases, circulating in the blood, which was produced in some way by the lack of the parathyroids. The injection of such guanidine base as guanidine hydrochloride into the rabbit, showed almost the same metabolic evidences in the urine and blood, accompanied by acidosis, which is considered by Morel (29) and some other workers to be the fun-

damental cause of tetany. Therefore, we may suppose that this condition of acidosis may influence the acid-base equilibrium, and it is necessary to estimate the phosphate and calcium content of the blood in guanidine tetany to compare with that in parathyroid tetany.

Methods.

Rabbits were used in this investigation. The content of phosphate and calcium was estimated before and after the administration of guanidine hydrochloride to compare the normal and tetanic condition. About 7 cc. of blood were drawn from the jugular vein and 1 cc. was used for the sugar estimation. The rest of the blood was kept over night for the separation of the serum. Marriott's (30, 31) methods for phosphate and calcium were employed, and his technique was followed.

In some cases after the administration of guanidine, the fat content was, apparently, very much increased. On filtering the calcium oxalate or ammonium magnesium phosphate through a Gooch crucible and washing with dilute ammonia, a colloidal solution is sometimes obtained which is very difficult to filter. This can be overcome by adding a small quantity of alcoholic ammonia to the crucible. This procedure results in no loss of phosphate or calcium. It is necessary to maintain uniform conditions of the animals in estimating the phosphate content of the normal rabbits. Oats and water only were given to the first group of rabbits, and oats, greens, and water to the second group throughout the investigation. In the first group the first blood was taken after the rabbits had been kept in the cage 24 hours, and after another 24 hours the injection of guanidine hydrochloride was performed. The second sample of blood must be taken after the acidosis becomes severe; but half of the rabbits of this group died before the blood was drawn. The data mentioned in Table I were obtained from samples taken from about 24 to 48 hours after administering the drug. A long continued acidosis was intended to be maintained in the second group of animals and for this purpose several small doses of guanidine were administered at about 1 day intervals and the rabbits were fed only greens and water after the injections.

In thirty-three estimates on twenty-eight rabbits, the normal content of phosphate varies from 2.1 to 5.6 mg. per 100 cc. of serum. This is a rather large variation when compared to the calcium content which varies from 10.5 to 13.0 mg. per 100 cc. of serum. The phosphate content may vary with the condition of the animal, the food, or with the environment. It may be parallel to the hydrogen ion concentration of the blood. The rabbits which had albumin in the urine show a rather high content of phosphate in the serum; Rabbit 4, which had heavy albuminuria and was pregnant, showed a high content; No. 5 also had heavy albuminuria and a high content of phosphate in the serum. Rabbit 1 was examined on three different days. The first estimate was 3.5 mg., but the third was the highest normal figure obtained—5.6 mg. This shows how variable is the normal figure for phosphate in the blood although this animal had albumin in the urine. We may consider that the normal content of phosphate in normal rabbits is from 2 to 4 mg. per 100 cc. of serum and that when above 4 mg. we may consider we have an acidosis. None of the rabbits employed in this investigation had sugar in the urine.

After the injection of guanidine almost all animals showed an increased content of phosphate in the blood and in some cases, Nos. 14, 22, 2, and 3, the amount was increased to five times the normal figure. Rabbit 14 was in moribund condition when the sample of blood was taken, 51 hours after guanidine injection; the same was true of No. 22, 25 hours after injection; No. 2, 22 hours after injection; No. 3, 15 hours after injection. All of these four rabbits showed increases about five times the normal. From these experiments the fact is established that in guanidine tetany the phosphate content of the blood is in almost all cases increased except Nos. 6, 12, and 5, but even here the values kept above what we consider the highest normal limits.

Another important evidence is the decreased calcium content, though it is not so marked. In Table I we have arranged in descending magnitude the phosphate content after guanidine injection. It may be noted that in all cases in which the phosphate reaches 6 mg. in 100 cc. of serum there is a decrease in calcium except No. 13 in which the phosphate rose so high that there was practically no change in calcium. The second speci-

TABLE I.

Phosphate and Calcium Content in Serum before and after the Administration of Guanidine Hydrochloride.

Rabbit No.	Albumin in urine.	Guanidine per kg. of body weight.	Phosphate and calcium content, and sugar before and after injection of guanidine.					
			Before.			After.		
			Phosphate.	Calcium.	Sugar.	Phosphate.	Calcium.	Sugar.
		gm.	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
14	−	0.27	4.0	12.0	0.133	18.2	8.7	0.072
22	−	0.15	3.0	12.6	0.133	16.0	10.0	
2	−	0.25	2.1	12.0	0.105	11.3	8.5	0.071
3	−	0.20	2.1	11.1	0.120	10.7	8.8	0.051
13	−	0.20	3.6	11.0	0.149	9.8	12.6	0.059
15	−	0.30	4.0	13.0	0.100	9.0	8.6	0.053
9	−	0.27	2.8	12.0	0.100	8.6	10.4	0.056
19	−	0.25	2.1	11.8	0.120	7.8	10.4	0.047
11	++	0.30				7.4	10.2	0.043
10	+	0.30				6.8	10.2	0.055
4	+++	0.20	4.0	11.0	0.087	6.0	11.2	0.085
27	−	0.15	2.4	12.4	0.125	6.0	11.0	0.053
7	−	0.30	3.8	11.0	0.111	5.4	12.0	0.083
6	++		4.0	12.4	0.143			
6	++	0.30	4.6	12.6	0.100	4.6	12.6	0.100
12	++	0.30	3.4	12.6		4.3	12.0	0.059
5	+++	0.33	4.5	10.5	0.091	4.0	11.0	0.084
1	++		3.5	11.1	0.111			
1	++		5.1	11.5	0.125			
1	++	0.20	5.6	10.8	0.125			
8	+	0.20	4.8	12.0	0.130			
17	−	0.20	2.8	12.8	0.109			
16	++	0.20	3.0	12.7	0.111			
18	++	0.20	3.0	12.4	0.125			
20	+	0.15	4.2	12.6	0.107			
21			3.2	11.0	0.143			
30	−	0.15	3.4	12.2	0.130			

men of blood was drawn 26 hours after injection and the rabbits manifested distinct tetanic symptoms and also were much more sensitive than the other animals. The phosphate increased but was not followed by the decrease in calcium. In general the increased phosphate is accompanied by a decrease in calcium and

it is clear that the rise in phosphate is followed by the fall in calcium from the fact that a rise in phosphate has been observed while the calcium remains normal. However, a fall in calcium with a normal phosphate has never been observed. More will be said of this in the discussion of the second group of rabbits.

In Table II is shown the ratio of phosphate and calcium before and after administration of guanidine injection. In Rabbit 2 the increase in phosphate over the normal before injection was 538 per cent, and in all other rabbits except Nos. 5 and 6, there was a distinct increase. The calcium in some cases decreased to 71 per cent of its initial values, and the others all decreased with the exception of a few cases. In normal conditions the ratio of calcium to phosphate varies from 5.7 to 1.9, but after guanidine injection it decreases to 2.8 to 0.5. If we exclude the "normal" rabbits showing albuminuria, the normal value of $\frac{\text{calcium}}{\text{phosphate}}$ is 3.0 or over. Therefore, we may say that in almost all cases after guanidine injection the ratio $\frac{\text{calcium}}{\text{phosphate}}$ decreases.

In the second group of rabbits we intended to secure more definite information on the decreased calcium content of blood following increased phosphate content. It was necessary to keep animals for a longer time, and for this rather small doses of guanidine were given occasionally and blood specimens were drawn at intervals. Nine rabbits were tried in this group, and in only a few cases were satisfactory results obtained. In Rabbit 23 (Table III) the normal phosphate content was 3.2 mg. and the calcium 12.1 mg. per 100 cc. of serum. After several injections of the drug, the phosphate rose to 5.2 and the calcium decreased to 6.6, about half of its normal value. The small injections of drug were continued and in the last specimen from this rabbit the phosphate decreased to 4.6 and the calcium was again restored toward normal though not reaching the true normal level. This shows that as the acidosis subsides the calcium is restored. Rabbit 24 shows almost the same phenomena though the decrease in calcium is not so marked as in the above case. In this rabbit the last specimen of blood contained less phosphate than former specimens but the calcium was not restored to normal; rather it decreased

TABLE II.

Relation between the Phosphate and Calcium Contents before and after Injection of Guanidine and the Ratio of Calcium to Phosphate before and after Injection of Guanidine.

abbit No.	Ratio of phosphate and calcium contents after, to the phosphate and calcium contents before injection of guanidine.		The ratio of calcium to phosphate before and after injection of guanidine.	
	$\frac{\text{Phosphate (after)}}{\text{Phosphate (before)}}$	$\frac{\text{Calcium (after)}}{\text{Calcium (before)}}$	$\frac{\text{Calcium}}{\text{Phosphate}}$ (before).	$\frac{\text{Calcium}}{\text{Phosphate}}$ (after).
14	4.55	72	3.0	0.5
22	5.33	79	4.2	0.6
2	5.38	71	5.7	0.7
3	5.10	79	5.3	0.8
13	2.72	114	3.1	1.3
15	2.25	66	3.2	1.0
89	3.07	87	4.3	1.2
19	3.71	88	5.6	1.3
11				1.4
10				1.5
4	1.50	102	2.8	1.9
27	2.50	89	5.2	1.8
7	1.44	109	2.9	2.2
6			3.1	
6	1.00	100	2.7	2.7
12	1.26	96	3.7	2.8
5	0.89	105	2.3	2.8
1			3.2	
1			2.3	
1			1.9	
8			2.5	
17			4.1	
16			4.2	
18			4.1	
20			3.0	
21			3.4	
30			3.6	

slightly. This shows that phosphate and calcium do not change distinctly parallel and supports the view that the change of phosphate is the preliminary change and that the calcium decrease or restoration is a secondary phenomenon following acidosis. The results of Rabbit 25 substantiate this theory; the first specimen of blood after injection of the drug showed an increase in phosphate

TABLE III.

Phosphate and Calcium Content in Serum before and after the Administration of Guanidine Hydrochloride.

Rabbit No.	Day and time.	Guanidine per kg. of body weight.	Phosphate and calcium content before and after injection of guanidine.		Sugar.	Remarks.
			Phosphate.	Calcium.		
		gm.	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	
23	1st, 3 p.m.		3.2	12.1	0.125	Wt., 1,660 gm.; moderate albumin but no sugar in urine.
	2nd, 3 "	0.1				
	3rd, 2 "	0.05				
	4th, 8 a.m.	0.05				
	1 p.m.		5.2	6.6	0.123	
	5 "	0.05				
	5th, 8 a.m.	0.05				
	2 p.m.	0.05				
	3 "		4.6	9.5		Animal killed.
24	1st, 4 p.m.		3.8	12.0	0.120	Wt., 1,460 gm.; very faint trace of albumin but no sugar in urine.
	2nd, 3 "	0.1				
	4th, 2 "	0.05				
	5th, 8 a.m.	0.05				
	2 p.m.		5.8	10.0	0.105	
	5 "	0.05				
	6th, 8 a.m.	0.05				
	2 p.m.	0.05				
	7 "		4.4	9.7	0.100	Animal killed.
25	1st, 2 p.m.		2.9	11.6	0.109	Wt., 2,520 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	4th, 5 p.m.	0.08				
	5th, 5 "	0.03				
	6th, 11 a.m.		4.9	11.2	0.091	
	5 p.m.	0.07				
	7th, 2 "				0.122	
	8th, 10 a.m.	0.10				
	9 p.m.		2.8	10.0	0.120	Moribund; killed.
26	1st, 3 p.m.		3.2	12.3	0.137	Wt., 2,220 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	4th, 5 p.m.	0.08				
	5th, 5 "	0.03				
	6th, 11 a.m.		4.8	12.0	0.125	
	5 p.m.	0.07				
	7th, 3 "		6.8	6.0	0.143	Animal killed.

TABLE III—*Concluded.*

Rabbit No.	Day and time.	Guanidine per kg. of body weight.	Phosphate and calcium content before and after injection of guanidine.		Sugar	Remarks
			Phosphate.	Calcium.		
		gm.	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	
28	1st, 4 p.m.		2.2	12.2	0.112	Wt., 2,920 gm.; trace of albumin but no sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 2 p.m.	0.08				
	6th, 11 a.m.		4.4	11.0	0.137	
	4 p.m.	0.07				
	7th, 10 a.m.	0.07				
	8th, 11 "		4.0	9.7	0.115	
	4 p.m.	0.10				
	9th, 9 a.m.	0.05				
	2 p.m.		4.0	10.5	0.100	Died 2 hours after last sample.
29	1st, 3 p.m.		3.6	12.0	0.125	Wt., 1,990 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 4 p.m.	0.08				
	6th, 4 "		3.0	10.2	0.122	
	5 "	0.07				
	7th, 4 "	0.10				
	8th, 9 a.m.	0.07				
	3 p.m.		4.3	11.0	0.117	Found dead 10th day, 8 a.m.
	9th, 3 "	0.10				
31	1st, 2 p.m.		4.0	11.4	0.111	Wt., 1,480 gm.; trace of albumin but no sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 10 "	0.10				
	6th, 12 m.		5.8	10.0	0.102	Killed.
32	1st, 3 p.m.		3.6	11.6	0.125	Wt., 2,150 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 10 "	0.10				
	6th, 3 p.m.		3.5	10.0	0.120	
	4 "	0.08				
	7th, 9 a.m.	0.07				
	4 p.m.		4.2	12.0	0.105	
	8th, 11 a.m.		6.0	10.4	0.120	Killed.
21	1st, 4 p.m.		6.2	8.0	0.139	Wt., 2,040 gm.; trace of albumin in urine.
	3rd, 9 a.m.					
	10 "		6.4	7.7	0.270	Died with convulsion.

from 2.9 to 4.9, but calcium still remained normal. In the second specimen after injection, the phosphate decreased but calcium was less than in the former specimen. Rabbit 26 distinctly substantiates the above theory; the first specimen after guanidine administration shows a rise in phosphate from 3.2 to 4.8, and calcium is unchanged; in the last specimen phosphate rises to 6.8 mg., and the calcium decreases to 6.0, about half the content of the normal. Rabbits 28 and 29 also show a similar behavior though not so distinct.

Rabbit 21 of this group had spontaneous acidosis. This rabbit was very sensitive and excitable. The blood before injection of guanidine contained 6.2 mg. of phosphate per 100 cc. of serum, which is much higher than in normal rabbits, and the calcium was 8.0 mg. After a small dose of guanidine the animal showed a more severe dyspnea than usual. Such a small dose—1.2 mg. per kilo of body weight—is not enough to induce such severe symptoms in normal rabbits. The rabbit died in convulsions 1 hour after the injection. The blood drawn at death contained 6.4 mg. of phosphate and 7.7 mg. of calcium, which are practically the same values as those before injection of guanidine in this rabbit, and so we may say that the drug had no influence on the calcium and phosphate in this short time. These facts strengthen the view that the acidosis plays an important part in the reduction of calcium in the blood.

DISCUSSION.

From the results of the present experiments, and of previous papers on guanidine tetany it is plain that following muscular activity a severe acidosis appears and that phosphate is retained in the body.

In the present experiment the acidosis became severe and the phosphate reaches a value above 6.0 mg. and is sustained, while the calcium may be decreased in these cases of guanidine tetany.

In the abundant literature on calcium in tetania parathyreopriva and idiopathic tetany some writers report a distinct decrease of calcium in the tissues and blood together with an increased excretion, while other authors found no appreciable change from the normal. Likewise in our experiments on guanidine tetany some cases show a distinct decrease in calcium while some remain

normal. We may conclude that in parathyroid tetany the decrease of calcium is not constant, and we may also suggest that the calcium decrease is not the fundamental cause of tetany, but it may be a secondary phenomenon of altered metabolism. Here we may call attention to the work of MacCallum and Voegtlin (13) who state:

"It was shown that the transfusion of blood from a normal dog will suppress the symptoms of tetany when a sufficiently large amount is introduced. Emulsion of brain substance was injected in the same way but the effect upon the tetany was so indefinite that it was questioned in that paper whether the action of the emulsion was any more marked than would have been that of so much salt solution or emulsion of any other organ from a normal dog. Emulsions of the parathyroid gland seemed to have a definitely specific effect but that appears only after the lapse of some hours whereas the effect of bleeding and infusion of relatively large amounts of salt solution or blood (200 to 400 cc.) from a normal dog has an almost immediate effect in suppressing the tetany. We could find no other explanation of this phenomenon than that some poisonous material, not destroyed or present in larger amounts than physiologically, on account of the absence of the parathyroid glands, was circulating in the blood."

In accord with the toxin theory, the increased content of guanidine was recently found. After injecting this toxin into animals they manifest almost the symptoms of tetany and also physiological and metabolic evidences, as is shown in the previous work and in the present experiment. We may now conclude that the true cause of tetany is the increased content of guanidine resulting from a disturbance in the function of the parathyroid. Another question naturally arises: How does the calcium injection temporarily abolish the tetany? We may imagine that in tetany the calcium of the tissues and blood may be inactivated or precipitated and excreted and that when soluble calcium is replaced or supplied to the normal content, the excitability of the nerve is reduced. Beside the work of Sabbatani (10), that of Loeb (32), Friedenthal (33), and also Garrey (34) in which is shown that when skeletal muscles are immersed in solutions which precipitate calcium less electrical stimulus is required to cause a contraction, supports the above view. MacCallum (35) also reported that when certain salts which precipitate calcium are administered the peristalsis of the intestine is increased. This also supports the view that the calcium content of the brain is instru-

mental in the excitability of the muscles. Starkenstein (36) reported that the introduction of phosphate and citrate in the form of sodium salts provokes muscular twitching. The fact is that the salts mentioned are precipitants of calcium. Quest (37) and many others made the interesting observation in feeding experiments that animals fed with calcium-free food or a diet with diminished calcium, showed an electrical hyperexcitability.

Recently MacCallum, Lambert, and Vogel (38) reported that in perfusion experiments in which dialyzed blood poor in calcium was perfused through the isolated extremity an extreme hyperexcitability was produced, quite like that observed in tetany. When the isolated limb was perfused with normal blood there was no hyperexcitability produced. They also established the fact that when tetanic animals were bled, and the blood was replaced by normal blood, the tetany was relieved and the hyperexcitability was lowered, though when dialyzed blood poor in calcium was replaced there were practically no changes. From this they support the deficiency of calcium theory of tetany. From the above works we may judge that in tetany the diminished calcium content of the brain is accountable for part of the hyperexcitability of the nerves, and replacement of the soluble calcium only diminishes part of the excitability in tetany. In other convulsive diseases, such as eclampsia and uremia, the calcium excretion is increased and the calcium content of the brain diminished (11). In tetany, however, the mechanism for the diminution of calcium in the tissues may be attributed to the acidosis since acid administration to animals causes an increased excretion of calcium (39) and magnesium, though this has not been so definite (40) until the present time.

Relation between Sugar and Calcium Content in the Blood in Guanidine Tetany.

In a preceding paper (41) we demonstrated that in guanidine tetany the content of sugar in the blood was slightly increased for the first few hours, especially after a large dose of the drug. This increase comes from the developing dyspnea caused by the disturbance of the respiratory center by the increased hydrogen ion concentration incident to the developing acidosis. On the contrary, several hours after the injection of the drug there appears a

hypoglycemia, while in some cases the blood sugar falls to half the normal quantity. In the present investigation we estimated the sugar in the blood simultaneously with the phosphate and calcium. The marked hypoglycemia was also produced in the first group of rabbits, half of them showing a decrease of half the normal. Comparing the calcium and sugar content in the blood of the same animals, it is clear that there is no relation between these two components of the blood. In Nos. 13, 12, and 27 the sugar decreases to half of the normal value while the calcium maintains the normal level.

Underhill and Blatherwick (42, 43) found that in parathyroid tetany the sugar content is markedly decreased and that the normal level can be restored by the injection of soluble calcium. From this fact they concluded that calcium plays an important rôle in maintaining the normal blood sugar level. In a previous (2) paper on guanidine tetany, we showed that the blood sugar could not be restored to normal by the injection of calcium lactate.

This hypoglycemia may be different from that in parathyroidectomy in which the increase of guanidine is more gradual than in guanidine tetany. Moreover, rabbits used in our experiments are less resistant to acidosis than the dog (44), and also the assimilation limit for injected glucose is lower in the rabbit (45) than the dog. These facts may explain the failure to restore the normal blood sugar level.

In looking over the literature on the influence of calcium on carbohydrate metabolism, we see the production of diabetes with calcium-poor diet by von Bunge (46), and the work of Underhill and Closson (47) on checking the glycosuria caused by continued administration of sodium chloride by the intravenous injection of calcium chloride. It is also reported that calcium administration inhibits the glycosuria caused by epinephrine (48), and the administration of sodium salts such as the oxalate or phosphate which precipitates calcium provokes the hypoglycemia (49, 50).

In considering the above facts it appears true that calcium plays an important rôle in regulating blood sugar content, but whether this is direct or indirect action is not known. In the second group of rabbits in Table III the blood sugar content shows no change even in cases where the calcium decreases to half of its normal content, as in Rabbits 23 and 26. In Rabbit 21, which

had a long continued acidosis, calcium diminished markedly, but the blood sugar was practically unchanged. On the other hand, after guandine injection in this animal the first specimen showed a high hyperglycemia caused by the dyspnea.

From this evidence we may consider that calcium indirectly influences the sugar-regulating mechanism. Here we recall that in parathyroidectomized animals the assimilation limit for dextrose introduced by mouth or subcutaneously (51, 52, 53, 54) is lowered. This may indicate that the oxidation process is lessened in this condition. It might be correlated with the condition of acidosis since this is supported by the work of Chvostek (55) and Munk (56) in which they found that the oxidative power of the body is lessened in acid intoxication. This may induce the hypoglycemia in tetany.

SUMMARY.

1. The content of phosphate in the serum of normal rabbits runs from 2 to 4 mg. per 100 cc. of serum. In guanidine tetany caused by a large dose of the drug it increases in some cases to five times the normal and in almost all cases it is somewhat increased. In most cases in which several small doses were administered the phosphate increases though it remains normal in a few cases.

2. The normal calcium content of rabbit blood varies between 11 and 13 mg. per 100 cc. of serum. In guanidine tetany where the phosphate increased in the blood, the calcium decreased somewhat later. In mild, long continued, acidosis, the calcium content decreased to about half the normal value.

3. The ratio calcium : phosphate in the blood is decreased in almost all cases of guanidine tetany.

4. In the first group of rabbits, in which was injected a large dose of guanidine, hypoglycemia, with or without diminution of calcium in the same sample of blood, was manifested, whereas the phosphate content rises distinctly above the normal in all cases. In the second group of rabbits, injected with small doses at intervals, the sugar content of all specimens remained unchanged even in cases in which the calcium diminished distinctly to a value half of the normal. A high phosphate content accom-

panied the low calcium. From these facts we may deduce the proposition that calcium is not concerned in the direct control of carbohydrate metabolism in guanidine poisoning but that some other intermediary metabolic change plays a part in this phenomenon. The hypoglycemia induced by guanidine is accompanied by acidosis. Long continued acidosis so alters the metabolism that it is difficult to induce hypoglycemia.

CONCLUSION.

The administration of guanidine to the animal body induces a condition of severe acidosis with the retention of phosphates, a decrease of calcium in the blood, and a hypoglycemia. These phenomena are also observed in tetania parathyreopriva. Further, the symptoms and all other physiological evidences in guanidine tetany are almost the same as in tetania parathyreopriva. Since the discovery of the large increase of guanidine bases in tetania parathyreopriva and idiopathic tetany, it is possible that the fundamental cause of tetany is the increased formation of guanidine nitrogen brought about by the disturbance of the function of the parathyroid.

The author acknowledges his indebtedness to Professor F. P. Underhill for his advice and interest throughout the course of this work.

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VITAMINE STUDIES. II.

DOES WATER-SOLUBLE VITAMINE FUNCTION AS A CATALASE ACTIVATOR?*

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INTRODUCTION.

In a recent paper (1) we have attempted to show that avian polyneuritis is accompanied by a lowering of the catalase activity of the liver, kidney, pancreas, heart, breast muscle, lung, and blood. We also pointed out that the catalase content of tissues, in polyneuritic pigeons which had received water-soluble vitamine prepared from wheat embryo, showed a tendency to return to normal shortly after the administration of the food hormone which McCollum and Kennedy (2) have designated as "water-soluble B." It was also observed (1) that pigeons which are relatively immune to polyneuritis possess tissues, even under conditions of inanition, which are practically normal in their catalase content.

We have therefore stated that an apparent relationship exists between oxidative processes and vitamine storage in avian polyneuritis. Birds which apparently had no vitamine became polyneuritic and possessed tissues low in this oxidizing enzyme. Inasmuch as the feeding of water-soluble B brought about normal catalase content of tissues, accompanied by improvement in the condition of the bird, it would seem reasonable to suppose that the vitamine had more or less to do with the oxidative processes. Where pigeons have lived for a considerable time on diets of polished rice and have been found to possess no symptoms of

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polyneuritis, and yet have been found to possess tissues which are normal in catalase, although the birds have lost in body weight, we have ventured the conclusion that these birds have been able to store larger quantities of water-soluble B in their tissues, previous to the rice diet, than the more susceptible pigeons were able to do.

Inasmuch as the water-soluble vitamine evidently causes increase in catalase production, we have conducted a few preliminary determinations to ascertain whether the stimulating action is direct or indirect.

Burge, Kennedy, and Neill (3) have shown that thyroid feeding affects the catalase activity of tissues, and Burge (4) has shown that this is also true for many food materials. Alcohol (5), when introduced into the stomach, caused an increase in blood catalase, but when alcohol was introduced directly into the blood, a decrease in blood catalase occurred. Kennedy and Burge believe (6) that the major portion of blood catalase arises in the liver, where it is stimulated to activity, or greater production at least, by pancreatic hormones, for pancreatectomy accomplished a lowering of blood catalase, while electrolytic stimulation of the splanchnic nerves caused a pronounced increase. Loevenhart (7) noted an increase in the output of oxygen when pancreas and liver and muscle and liver were combined in hydrogen peroxide. More recently Burnett (8) has made quantitative measurements on various tissues and has found that blood and liver accelerated the catalase activity of muscle tissue, and he therefore advanced the tentative explanation that the acceleration was due to an internal secretion which acts as a catalase activator. In order to ascertain whether our vitamine extracts possessed this property of direct stimulation, the following experiments were conducted.

EXPERIMENTAL.

A polyneuritic pigeon was killed in the early stage of the disease, and the liver, which weighed 4.806 gm., was washed free of blood with 0.9 per cent salt solution. The liver was minced and suspended in distilled water for 3 hours. The water suspension was filtered and washed, the filtrate being made to 100 cc. volume. Portions of 5 cc. each of the liver extract were placed in the cat-

alase apparatus described in a previous paper (1), and the oxygen measurements were made at the end of 10 minute periods. The bottle in every case contained 100 cc. of neutral hydrogen peroxide (3 per cent) and was agitated at the rate of 110 double shakes per minute. All measurements were made at 20°C. and 740 mm. pressure. Averages of several determinations on the 5 cc. samples of liver extract gave the following results:

Sample No.	cc.
1	300
2	305
3	307
4	302
Average	303

Three different vitamine extracts were used in this work, all of which had shown curative properties in our work on pigeons. Vitamine Extract I was prepared from wheat embryo and evaporated in the presence of dextrin, yielding cream-colored powder. Vitamine Extract II was also prepared from wheat embryo but was in the form of a concentrated syrup. Vitamine Extract III was a powdered sample prepared from corn pollen, with an admixture of dextrin.

None of the vitamine extracts showed any activity toward hydrogen peroxide. Determinations were made on the liver extract, using 5 cc. samples, except that the various vitamine extracts were introduced into the hydrogen peroxide bottle. All determinations gave negative results. Duplicate determinations on 5 cc. samples of the liver extract in the presence of vitamine extracts gave the following figures:

5 cc. liver extract	+	Vitamine Extract I	=	300	cc.	O_2
5 " "	"	+	"	"	II	=	305	"	"
5 " "	"	+	"	"	III	=	296	"	"
Average of liver extract alone				=	303	"	"

CONCLUSION

The results indicate that water-soluble B does not act as a direct activator of catalase, but instead probably (on account of its physiological properties) stimulates the organism to greater production of catalase.

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VITAMINE STUDIES. III.

OBSERVATIONS ON THE CURATIVE PROPERTIES OF HONEY, NECTAR, AND CORN POLLEN IN AVIAN POLYNEURITIS.*

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INTRODUCTION.

Since the United States Food Administration first showed the necessity of conserving the available supply of cane and beet sugar, much has been written to show that one way to conserve sugar would be to encourage consumption of larger quantities of syrups and honey. Much of the printed matter is designed for the general reader and, of course, many extravagant statements have been made, some of which have not accomplished the purpose for which they were written, but in the main, much real good has been attained.

In extolling the virtues of honey some writers in the past have pointed out that, although honey is not as economical as commercial sucrose, it is superior to ordinary sugar on account of its laxative action, fuel value, and medicinal properties. More recently honey producers have been asking whether honey contains vitamines. Inquiry shows that statements in the affirmative have been made in popular articles, but we have been unable, up to the present time, to find any record of investigational work in this field.

On account of the importance of this question at the present time, we have conducted experiments, which will be described

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later, attempting to prove or disprove the presence of vitamines in honey.

A study of the habits of the honey-bee shows that there are, generally speaking, two types of food used by the bee, floral nectar and pollen. The floral nectar is brought to the hive in the honey stomach of the bee and deposited in the cell where it is evaporated later to honey. Most of the pollen is carried from the flower to the hive in the pollen baskets or corbicula situated on the tibiæ of the third pair of legs (1); it is also transported to a much lesser degree by clinging as a dust to the body hairs. This pollen is moistened and packed into certain cells and is known as bee bread. Unpublished data in this laboratory indicate that the pollen is probably moistened with nectar or diluted honey. It is from this bee bread and honey that the larval food of the future generations is prepared.

In planning the experimental work it was felt that, should honey contain vitamines, there would be a greater possibility of finding the water-soluble vitamine in honey than its fat-soluble prototype, due to the very nature of honey which is high in water-soluble substances but low in fatty materials.

It was first pointed out by McCollum and Kennedy (2) and later corroborated by Drummond (3) that the substance found in seeds, seed embryos, thin leaves of plants, and potato juice, which cured avian polyneuritis and assisted in the general well being of growing animals, is probably one and the same substance. This has been called "water-soluble B." It was with the view of studying honey with reference to this vitamine that the following experiments were conducted.

EXPERIMENTAL.

The honey used in this experiment was a clear sample of strained honey obtained while basswood and white clover were in full bloom. The nectar was obtained largely from white clover, although basswood was in bloom at the time but was not being visited by many bees. This material was obtained by centrifuging the uncapped combs, newly filled with nectar, before time had elapsed for evaporation to take place. Our reason for using nectar was to study the effect of evaporation and "ripening" of honey upon its vitamine content, should vitamines be found to be present.

Microscopical examination of the nectar and honey showed a few grains of pollen to be scattered through the mass. Consequently it was thought that the water-soluble vitamine content of pollen should be taken into consideration also. Fortunately there were available for this work several pounds of corn pollen which had been collected for other experimental projects in this laboratory. It was assumed that corn pollen would be representative of other pollens in this regard and therefore it was used because of the quantity easily available.

On June 29, 1918, fifteen pigeons were placed on a diet of polished rice and these were fed, watered, and weighed in the same manner as those in preceding experiments (4). Pigeon 26 became polyneuritic on the 20th day of rice feeding and was fed about 20 gm. of basswood-clover honey (diluted) at about 9.30 a.m. At noon on the same day the bird was found dead. Similar results were obtained when Pigeon 27 became polyneuritic on the 22nd day, except that the bird did not die so quickly as Pigeon 26.

Funk and von Schönborn (5) have advanced the hypothesis that vitamines have a distinct effect on carbohydrate metabolism, and Vedder and Clark (6) are of the belief that fowls with high metabolic activity require larger amounts of vitamines and succumb more promptly to diets of polished rice.

Theiler and his coworkers (7) and Braddon and Cooper (8) found that forced feeding brought on the disease much more quickly than when the pigeon was allowed to starve itself partially. Our work is in agreement with this.

It would appear, therefore, that Pigeons 26 and 27 might have died because of overloading of the oxidative mechanism during a low vitamine feeding. Seidell (9) and Emmett and McKim (10) have shown that vitamines are adsorbed upon colloidal suspensions of siliceous earths, and this method has been used by them to separate the vitamines from other materials. With this in mind, 2 pounds of basswood-clover honey were dissolved in water and shaken for about an hour with Lloyd's reagent,[1] after which the siliceous earth was filtered and washed until it was no longer

[1] This highly adsorptive siliceous earth was generously supplied by Professor Uri Lloyd of Cincinnati.

sweet to the taste. This paste was transferred to a graduated flask and made to 200 cc. volume with water. 10 cc. portions, representing 45 gm. of honey, were fed at each feeding when needed.

Pigeon 23 showed symptoms of polyneuritis on the 19th day and was fed 10 gm. of honey in the morning. By 3 o'clock in the afternoon the bird was acutely polyneuritic with the head drawn over its back. At 5 o'clock the same afternoon a 10 cc. portion of the Lloyd's preparation from honey was administered. The next morning the pigeon was walking weakly about the cage. Toward evening the bird was much stronger, at which time another 10 cc. portion of the preparation was fed. Thereafter ground rice was fed daily with a 10 cc. portion of the Lloyd preparation, and no particular change could be noted. The bird remained on its feet but walked with difficulty. After 10 days a small amount of vitamine extract of wheat embryo was fed to this pigeon and recovery was immediate, showing that the vitamine content of the honey was relatively low compared to that in the embryo of wheat.

Similar results were obtained on Pigeon 20, with the Lloyd preparation from honey, except that the curative properties of the preparation were even less evident than in the case of Pigeon 23.

The vitamine from 2 pounds of nectar was adsorbed on Lloyd's reagent, and made to 200 cc. volume. In this case also, 10 cc. represented 45 gm. of nectar. This preparation was fed to Pigeons 13, 16, 17, 18, 21, and 22 as soon as they became polyneuritic. The pigeons were forcibly fed as soon as they refused the polished rice.

We were unable to see improvement in any of the birds receiving the nectar preparation; all birds so treated died almost immediately or were cured by changing from this diet to one high in water-soluble vitamine.

The vitamine extract from the pollen was a dry powder prepared by extracting pollen in hot 50 to 65 per cent alcohol and evaporating this on dextrin at a relatively low temperature over a steam-heated sand bath in the air current from an electric fan.

Without going into detail regarding the efficiency of the vitamine extract prepared from corn pollen, it is sufficient to say that all polyneuritic birds which received this preparation recovered very quickly. In the case of Pigeon 15 we were able to bring on symptoms of polyneuritis three successive times and then to

cause the symptoms to disappear each time by a pinch of the pollen extract. Pollen extract was also fed to Pigeons 16 and 18 which were dying, due to the lack of vitamine in the Lloyd preparation from nectar. In both of these cases the pigeons improved sufficiently to fly to their perches within 36 hours.

CONCLUSIONS.

Honey contains a small amount of water-soluble vitamine, but the amount is negligible. It is probable that the slight antineuritic properties of honey are overbalanced by the large amount of carbohydrate present, for the presence of vitamines was made evident only upon removing the sugars by adsorbing the vitamines upon siliceous earth. There was very little evidence of the presence of water-soluble vitamine in the dilute unevaporated nectar. Corn pollen is relatively rich in water-soluble vitamine and it is possible that the small amount of this in honey may have its origin in the pollen of flowering plants, either in the form of pollen grains adventitiously present, or in the digested products, which might be introduced by the salivary juices of the bee.

I am indebted to Mr. Ferdinand A. Collatz for assistance in feeding and weighing the pigeons.

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A METHOD OF MEASURING THE ELECTRICAL CONDUCTIVITY OF LIVING TISSUES.

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(Received for publication, October 28, 1918.)

The purpose of this paper is to describe the method employed by the writer to determine the conductivity of living tissues.

This method originated as the result of studies on protoplasmic permeability. Early in these investigations the writer realized that quantitative experiments were essential to progress and undertook to develop accurate methods of measurement. The first experiments in this direction were made by plasmolysis. In connection with this the question arose whether salts penetrate as ions or as undissociated molecules. It is evident that the greater the permeability of the protoplasm, the more easily will an electric current pass through it. Hence if we can measure the electrical conductivity of the protoplasm we can measure its permeability to ions. For this purpose determinations of the conductivity of plant tissues were undertaken. The method which was devised for this purpose was developed without knowledge of methods which had previously been used for animals and differed from them. It proved to be accurate and convenient and was extensively used in subsequent investigations.

The method consists in placing the tissues between two electrodes and measuring their electrical resistance by means of a Wheatstone bridge. It is evident that some of the current must pass between the cells, flowing in the intercellular substance or spaces, but it has been shown by experiments on *Laminaria*[1] that a part of the current flows through the protoplasm.

It is desirable that the intercellular space or substance shall be constant in amount. This is the case in tissues, such as those of *Laminaria*, where the cell walls are of a firm consistency and

[1] Cf. Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 485.

do not change during the experiment.[2] On the other hand many flowering plants present difficulties, since the spaces between the cells are largely filled with gas, which is displaced to a varying extent when the tissue is placed in a solution, with the result that the conductivity is altered. In such cases we must select material in which the displacement is very slow or else we must get rid of the gas at the start by submerging the tissue and evacuating by an air pump.

As the writer's investigations were concerned with alterations in permeability it was necessary to provide for quick changes of reagents and for rapid penetration. This was accomplished by the use of thin sheets of tissue. For example it was found that when *Laminaria* was transferred from sea water to sea water diluted with an equal volume of distilled water, diffusion was practically completed in 5 minutes; this was also the case with the other material used in his investigations.

It is desirable that the thin sheets of tissue should be stiff enough to be handled easily and that they should not adhere to each other but should tend to separate spontaneously (this is assisted by a slight curvature of the pieces).

The cell walls should be thin and free from spaces containing gas. The material should be able to stand laboratory conditions and the manipulation required by the experiments. It is desirable that it should be available throughout the year. All these requirements are so admirably fulfilled by the marine alga *Laminaria agardhii* (a common kelp of the Atlantic coast) that it was largely used. It forms fronds several feet in length, 3 to 6 inches wide, having somewhat the consistency and thickness of a thin leather belt. It remains in normal condition in the laboratory for several weeks if kept in sea water (near 0°C.) and is not injured by the pressure and the weak electric currents to which it is subjected during the experiments.

Most of the experiments were made by means of the apparatus shown in Fig. 1. This will be called Type A in order to distinguish it from other types to be described later. It consists of two platinum electrodes,[3] A, sealed into glass tubes, B, which are

[2] Plasmolysis must be avoided since this increases the spaces between the protoplasmic masses.

[3] These are covered with platinum black.

filled with mercury and into which dip copper wires, C, which go to the Wheatstone bridge. These tubes are contained in electrode holders of hard rubber, D, through which pass a rod, E, and a long screw, F, by means of which the electrode holders may be drawn toward each other and held firmly in any desired position. This screw fits into an internal screw contained in the electrode holder at the right. This is not the case with the electrode holder at the left and in consequence this electrode holder is drawn toward the other only when the block, M, is fastened in place by the set screw, N, and the screw, F, is turned in the proper direction.

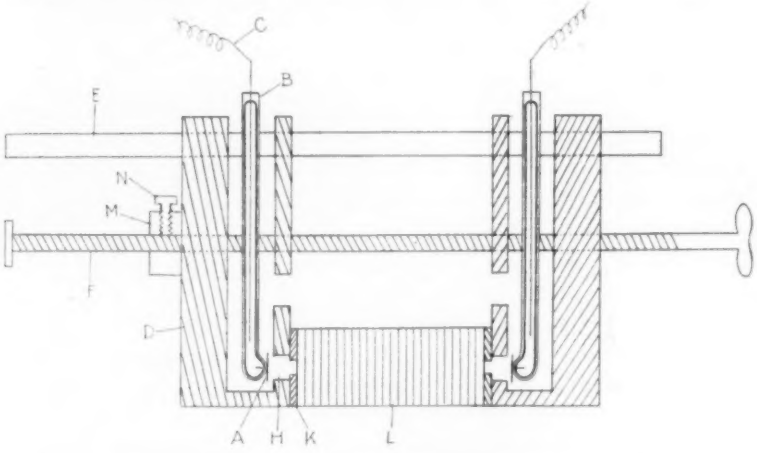

FIG. 1. Apparatus for determining the electrical conductivity of living tissue. The disks of tissue, L, are packed together like a roll of coins. At each end is a platinum electrode, A, fastened in an electrode carrier, D. By means of the screw, F, the electrode carriers can be drawn together, compressing the tissue and holding it firmly in place.

An end view of an electrode holder, D, is shown in Fig. 2. Its lower portion (which contains the platinum electrode) is shown inserted into a hard rubber support, G. The support is pierced by a series of seven holes (arranged in a circle as shown in the figure) each of which receives the end of a glass rod about 9 inches long, the other end of each rod being fastened in a similar support. The circle (dotted line) just inside the seven small circles represents a disk of tissue inserted between the glass rods with its

surface at right angles to them. The smaller circle, H, in the center represents an opening in the electrode holder through which the current passes from the platinum electrode to the disks of *Laminaria*. The arrangement is shown in Fig. 1, where H represents the opening and L represents the disks. Before reaching the disks the current passes through, K, (Fig. 1), a hard rubber disk (with an opening in the center) which provides mechanical support for the tissue.

The disks are cut from the fronds by means of a cork borer and have about the diameter and thickness of a silver quarter. They are packed together like a roll of coins (about 80 in all) and form a mass of just the right elasticity for the purpose of the experi-

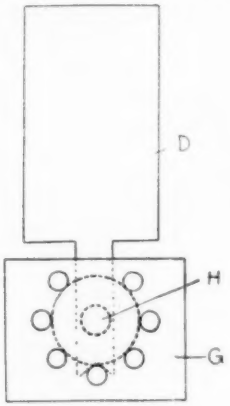

FIG. 2. Electrode carrier, D, seen from the end, resting upon a frame, G, in which are set glass rods (seen in section, as a series of circles) which hold in place the disks of tissue (seen in section as a dotted line).

ment. They are held firmly in place by the glass rods which surround them and by the electrode holders which press against them at either end. At the same time the spaces between the glass rods allow free circulation of liquid.

Each disk is placed in sea water as soon as it is cut;[4] from this the disks are transferred to the support G, which is submerged

[4] It was at first thought that cutting might injure the tissues at the edge of the disk sufficiently to interfere with the results, but experiments proved that this is not the case. Not only do the cells adjoining the cut surface live as long as those in the center of the disk but it is found that experiments (made by another method) on intact fronds give the same results as experiments on the cut disks.

in sea water. They are arranged inside the glass rods (being packed together, like a roll of coins, to form a cylinder) by means of forceps, and care is taken to see that no bubbles of air are caught between them or in the opening in K. The electrode holder is now placed in position and inspected to see that no air is caught in the space around the electrode or in the opening at H.

The electrode holders are now pressed against the ends of the roll of disks, the block, M, is firmly fastened by means of the set screw, N, and the screw, F, is turned until the electrode holders are tightly clamped against the roll of disks. The pressure used in this operation should be fairly uniform.[5]

The apparatus is now lifted out of the sea water[6] and allowed to drain[7] for a definite time (not over 1 minute) after which the resistance becomes practically constant.

The current passes for a short distance through sea water before reaching the disks. There is a film of sea water between each pair of disks and likewise a film around the cut edges. Otherwise the current passes only through the tissue.

As soon as the resistance has been measured the apparatus is replaced in sea water; the set screw, N, is loosened so that the electrode holders can be moved apart and the disks separated from each other by means of forceps. After standing for a few minutes in sea water the resistance is again determined. The disks are then separated as before and allowed to stand in sea water. This procedure is continued until it becomes evident that the resistance is practically stationary.[8]

The apparatus is then transferred to another solution (*e.g.*,

[5] It was at first thought necessary to use a dynamometer but it was found that the operator soon becomes so proficient as to make it unnecessary. The resistance is very little affected by variations in pressure.

[6] In the earlier experiments the resistance was taken with the cylinder submerged in sea water, and this may be preferable in special cases.

[7] Each support rests on a block of paraffin. Care must be taken that there is no conduction between the blocks; *e.g.*, along the wet surface of the table.

[8] Unless this is the case the material is rejected. With good material the resistance remains stationary for a long time (in one experiment it remained so for 10 days). See Osterhout, *Bot. Gaz.*, 1915, lix, 242.

NaCl 0.52 M) having the same conductivity (and temperature[9]) as the sea water. There should be at least 1,500 cc. of solution, contained in a shallow dish of glass or enameled ware. The disks are at once separated by means of forceps and thoroughly rinsed in the new solution, the whole apparatus being moved about in the dish to secure thorough mixing. By means of a medicine dropper the sea water around the platinum electrodes is thoroughly washed out. In some cases it is desirable to transfer to a second dish to ensure against contamination by sea water.

By this means a very rapid change is effected and, as the disks are thin, diffusion is soon completed (this is often the case in 5 minutes and should not in any event require more than 10 minutes). Since the outward diffusion of salts may take place at a different rate from the inward diffusion there may be an apparent rise or fall of resistance in consequence. This effect lasts but a short time and is found in dead as well as in living tissue. It is therefore easy to guard against error due to such causes.[10]

Certain additional precautions deserve mention. Only sound material should be chosen and disks should be cut from portions of the frond which are smooth and firm.[11] The best disks are those which curve slightly, so that when in the apparatus they spontaneously separate whenever the pressure is removed.

The resistance of the disks at the ends is much greater than that of those in the middle since the current spreads out after issuing from the small opening,[12] H, (Fig. 1) in the rubber disk. For this reason the best disks of tissue should be placed at the ends and their positions should not be changed. Care should be taken that they are not cut or injured by contact with the edges of the opening in the rubber disk.[13] The inequality between the

[9] All readings should be made at the same temperature or, if this is not practicable, should be corrected to the standard temperature. For the temperature coefficient see Osterhout, *Biochem. Z.*, 1914, lxvii, 272.

[10] Cf. Osterhout, *J. Biol. Chem.*, 1918, xxxvi, 489.

[11] Portions of the frond which are covered with reproductive tissue should not be used.

[12] It results from this that the resistance does not increase in direct proportion to the number of disks. If we plot the resistance as ordinates and the number of disks as abscissæ, we obtain a curve which is concave toward the base line. The curve is approximately logarithmic.

[13] These edges may be rounded by filing. A soft rubber disk may be placed between the hard rubber disk and the tissue.

disks at the end and in the center may be minimized by introducing at intervals rubber disks provided with openings in the center (Fig. 3). This is in many cases desirable and enables us to get a high resistance with less tissue.

Care must be taken to see that liquid does not leak out of the space around the electrodes while the apparatus is out of the liquid. If a leak should occur fresh liquid may be added by means of a medicine dropper. With a proper adjustment of the rubber disks and sufficient tissue to give elasticity no leakage should occur.

In regard to the accuracy of the readings it may be said at the outset that under favorable conditions successive readings on the same material do not vary more than 1 per cent from the average. This is as great accuracy as can ordinarily be hoped for in

FIG. 3. Hard rubber disks, A, alternating with disks of tissue, B (all seen in section).

biological work and there is no object in striving to get greater accuracy than this in the apparatus itself.

It is desirable to get a good minimum in the telephone and the insertion of capacity in the circuit may help to accomplish this. The frequency is also of some importance in this connection. The writer has found a thousand cycles convenient; this is furnished by an apparatus obtained from Leeds and Northrup.

The use of the ordinary lighting circuit (60 cycles) with a vibration galvanometer is recommended by Green.[14] An alternating current galvanometer may be used in connection with a recording device if desired.[15]

[14] Green, N. B., *Am. J. Bot.*, 1917, iv, 411.

[15] Cf. Weibel, E. E., and Thuras, A. L., *J. Ind. and Eng. Chem.*, 1918, x, 626.

For details regarding apparatus the reader is referred to the papers of Hibbard and Chapman,[16] of Washburn,[17] and of Taylor and Acree.[18]

We may now turn to another form of apparatus which may for convenience be called Type B. Fig. 4 shows one end of the apparatus, which consists of an electrode holder, A, and a series of glass cells, B, C, etc. The electrode holder consists of a glass tube provided with side arms for the admission of the electrode tube, D, (which is similar to the tube used in Type A) as well as

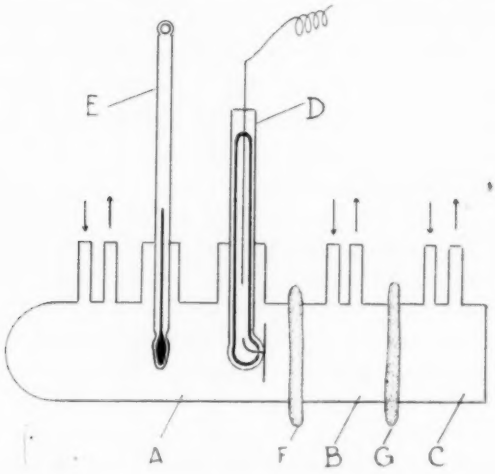

FIG. 4. Electrode carrier, A, consisting of a glass tube provided with a series of side tubes to hold an electrode tube, D, and a thermometer, E; also an inlet tube and an outlet tube. To the right two glass cells, B, C, each with an inlet tube and an outlet tube, with disks of tissue, F and G.

of a thermometer, E. In addition there is an inlet tube and an outlet tube by means of which the solution may be changed. Each of the glass cells, B, C, etc., has a similar inlet tube and

[16] Hibbard, R. P., and Chapman, C. W., *Michigan Agric. College, Technical Bull.* 23, 1915.

[17] Washburn, E. W., and Bell, J. E., *J. Am. Chem. Soc.*, 1913, xxxv, 174. Washburn, E. W., *ibid.*, 1916, xxxviii, 2431. Washburn, E. W., and Parker, K., *ibid.*, 1917, xxxix, 235.

[18] Taylor, W. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2415, and previous papers in the same *Journal*.

outlet tube. Each outlet tube has a rubber connection through which liquid can be discharged without wetting the cells. All of the inlet tubes are connected (by rubber tubing and a system of Y-tubes) to the same funnel so that all the cells can be filled simultaneously.

The edges of the glass cells are ground in a plane exactly at right angles to the long axis of the cell. When pieces of *Laminaria* are placed between them (as at F and G) and they are pressed together, a tight joint is formed. The series of glass cells (with pieces of material) and an electrode carrier at each end are placed in a V-shaped trough with rigid ends; at one end is a screw by means of which they can be forced together and held with any desired degree of pressure. At the places where the pieces of material are located, the trough is cut away so that they do not come in contact with it. Care is taken to keep the current from leaking along the trough (its surface is covered with paraffin).

The current therefore flows through the glass cells and through the pieces of material placed between them.

The advantages of this type of apparatus are: (1) the end pieces do not have more resistance than those in the middle; (2) the solutions may be changed without disturbing the material.

Types A and B may be combined by substituting disks of *Laminaria* for the glass cells.

Type C is shown in Fig. 5. It consists of two electrode carriers similar to those in Type A. The material is shown at M, its edges being completely surrounded by vaseline, V, V, so that the current cannot leak out. In many cases it is preferable to use chicle, grafting wax, or art gum in place of vaseline. The apparatus remains partly submerged (the water line being indicated at W, W), thus keeping the temperature more nearly constant. The solutions are changed by siphoning through the openings which admit the electrode tubes. This makes it unnecessary to unscrew and separate the electrode carriers during the experiment.

Type D is shown in Fig. 6. It permits the use of intact plants. One end of the plant is inserted into each of the cells A and B and held in place by a split rubber stopper. The cells A and B are filled with solution. The free portion of the plant is bathed in any desired solution until a reading is to be taken, when the

solution is allowed to drain off and the reading is made. Care should be taken to prevent the current from leaking through or around the stopper.

The part of the frond which is contained in the stopper and in the cell may be killed to lessen its resistance.

Material which is too soft to be handled in the manner recommended for *Laminaria* may be treated as follows: If it forms sheets or membranes it may be fastened to thin disks of hard

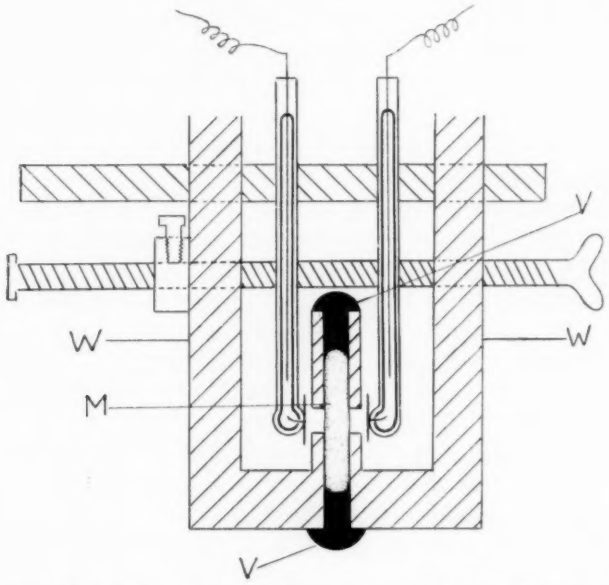

FIG. 5. Disk of tissue, M, the edges surrounded by vaseline, V, V, with an electrode carrier on each side.

rubber[19] provided with a central opening as shown in Fig. 7, in which A represents the rubber disk (seen in section), B the material, and C another disk of thin rubber or celluloid. These are fastened together by rubber bands, D. For this purpose three projecting knobs are provided as shown in the surface view at the left of Fig. 7. The disk is placed in the frame described under Type A, and the knobs fit in between the glass rods in the manner

[19] The edges of each piece of tissue are protected by vaseline.

shown in Fig. 7 (where the rods appear in section). Every other disk is turned upside down so that the knobs of adjacent disks

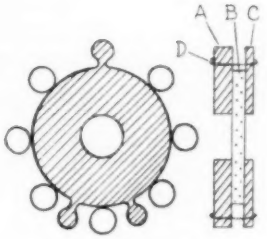

FIG. 7.

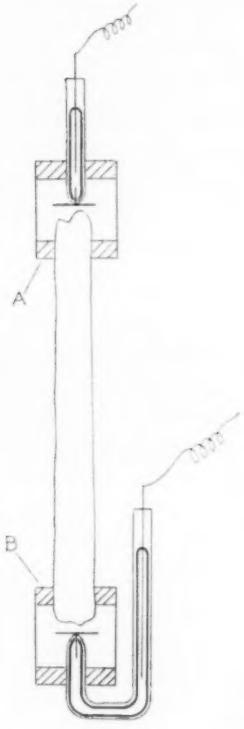

FIG. 6.

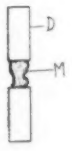

FIG. 8.

FIG. 6. Two glass cells, A and B, each provided with an electrode; a strip of tissue is stretched between them.

FIG. 7. A disk of hard rubber, A, one of tissue, B, and one of celluloid, C, tied together with rubber bands, D (all seen in section). Surface view at the left.

FIG. 8. Disk of hard rubber, D, with a mass of tissue, M, wedged in the central opening (seen in section).

do not touch and interfere with the close packing of the disks. The disks are treated precisely like the disks of *Laminaria* as described under Type A.

Material which cannot be handled in this way may be treated as shown in Fig. 8, where D represents a hard rubber disk with a central opening into which the material is tightly wedged. The disks are then handled like so many disks of *Laminaria*.

SUMMARY.

A method of measuring the electrical conductivity of living organisms is described which can be applied to pieces of tissue or to intact organisms. Under the most favorable circumstances measurements made by this method do not vary more than 1 per cent from the mean.

THE EFFECT OF THE MATERNAL INGESTION OF DESICCATED PLACENTA UPON THE RATE OF GROWTH OF BREAST-FED INFANTS.

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(*From the Department of Anatomy, Harvard Medical School, Boston.*)

(Received for publication, October 8, 1918.)

During a series of experiments designed to show whether or not the maternal ingestion of desiccated placenta would have an effect upon the chemical composition of the milk produced during the early stages of lactation,[1, 2] a comparison of the growth of the infants feeding upon the milk produced during its administration with the growth of those subsisting upon milk from mothers who had not been given the material,[3] led to the idea that the feeding of this substance to the mothers had an effect upon the rate of growth of their breast-fed infants entirely aside from the per cent change in the determined constituents.

Since the number of observed cases was too small to give more than the indication of a tendency, I decided to make a more extensive study of the matter, in order to determine definitely whether placenta tissue *per se*, when fed to nursing mothers, contains a substance or substances affecting the rate of growth of the breast-fed infants, it being a well known fact that milk may contain as such, or slightly changed, various substances, ordinarily foreign to its make-up, ingested by the mother.

The cooperation and courtesy of the staff and nurses of the Boston Lying-In Hospital made possible the carrying out of this plan, and the following is a brief report of the results.

In order to avoid complications in recording, all patients were given the desiccated placenta prepared as described in an earlier publication,[2] and in the same dosage, *e.g.* 10 grains in capsules,

[1] Hammett, F. S., *J. Biol. Chem.*, 1917, xxix, 381.

[2] Hammett, F. S., and McNeile, L. G., *J. Biol. Chem.*, 1917, xxx, 145.

[3] Hammett and McNeile, *Science*, 1917, xlvi, 345.

t.i.d. From these, only those mothers were chosen whose parturition course was normal, and only the weights of those infants are utilized whose sole source of nourishment was the maternal breast. The diet of the mothers and the method of weighing of the infants were the same as reported in the paper on the normal growth capacity of infants at this Hospital,[4] thus standardizing the experimental conditions for a study of this type.

In view of the results obtained in a study of the relationship between weight at birth and growth capacity,[4] and for purposes of comparison, the subjects of this experiment were also divided

TABLE I.

Comparison with the Normal of the Per Cent Change in Weight from the 1st Day of Infants Subsisting upon Milk Produced under the Influence of Maternally Ingested Desiccated Placenta.

Group.	Weight.	Day.											
		3		5		7		9		11		13	
		Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.
	lbs.												
A	5–6	−3.1	−4.0	−1.2	−3.3	0.6	−0.4	4.7	1.4	8.8	3.9	12.5	6.0
B	6–7	−4.1	−4.7	−2.1	−3.2	0.4	−1.1	3.0	0.9	5.2	2.8	7.9	4.4
C	7–8	−3.7	−4.4	−2.7	−2.7	0.1	−1.1	2.3	1.0	4.3	2.7	5.9	4.6
D	8–9	−4.5	−5.1	−3.0	−3.8	−1.3	−2.4	0.3	−0.6	2.0	0.9	3.3	2.1
E	9–10	−4.5	−7.2	−4.8	−7.1	−3.3	−5.9	−3.0	−4.7	−1.7	−4.0	−1.1	−2.9
F	10–11	−5.4	−7.4	−5.6	−7.3	−3.5	−6.4	−0.9	−5.6	0.9	−4.7	1.5	−3.6

into six groups according to weight at birth; and the weights recorded on the 1st, 3rd, 5th, 7th, 9th, 11th, and 13th days after birth were utilized for the data presented. The growth of 177 infants was studied during this period.

In Table I is given a comparison of the per cent change in weight of the two general sets of subjects. Fig. 1 is a graphic representation of the mean curve of each set.

The effect of the ingestion of the desiccated placenta by the mothers on the rate of growth of the breast-feeding infants is at

[4] Hammett, *Am. J. Physiol.*, 1918, xlv, 396.

once apparent. In each group not only is the postnatal decline in weight less in amount, but also the gain in weight after the preliminary loss is greater in every case on every day than that recorded for the normal groups, the mean increase over the normal per cent change in weight on the 13th day being over 60 per cent.

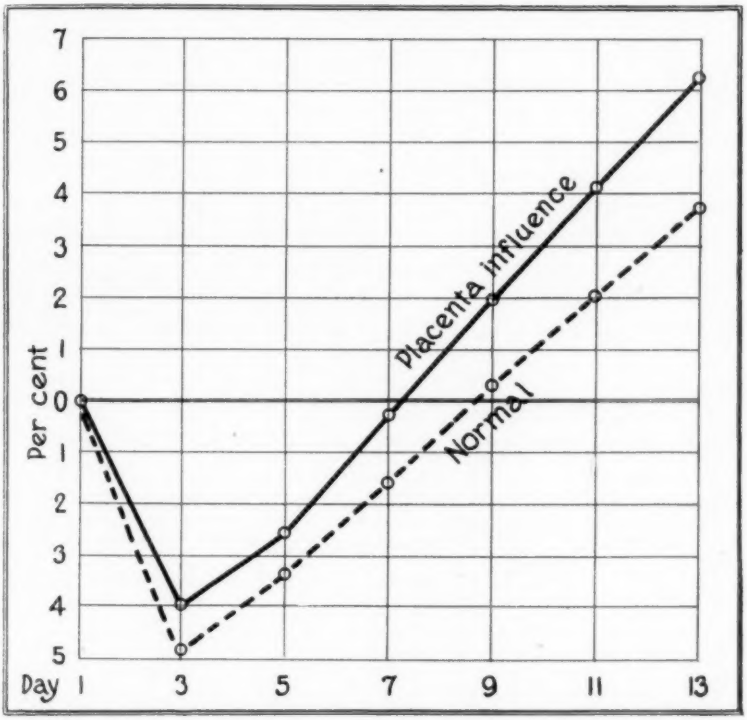

FIG. 1. Curves showing mean of per cent change in weight from 1st day of two sets of infants.

Supplementing these facts with an examination of Table II conclusively demonstrates that the rate of growth of breast-fed infants is enhanced by the maternal ingestion of desiccated placenta, for not only is the recovery to or over the initial weight generally more rapid, but the weight is almost uniformly greater.

The growth capacity of infants subsisting upon the milk produced during the administration of desiccated placenta is also increased. This is shown in Table III. That is to say, the maternal ingestion of dried placenta tissue so stimulates the tissues of the infants feeding upon the milk produced during this time,

TABLE II.

Comparison of the Per Cent Recovery to or over the Initial Weight of the Two Sets of Infants.

Group.	Weight.	Day.											
		3		5		7		9		11		13	
		Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.
	lbs.												
A.	5–6	17	19	17	29	58	50	83	62	100	75	100	82
B.	6–7	12	8	36	24	50	45	71	60	79	75	95	80
C.	7–8	0	12	27	24	54	39	78	60	87	74	89	78
D.	8–9	5	17	17	17	40	30	60	49	70	60	79	70
E.	9–10	0	2	22	5	33	15	33	20	33	30	55	35
F.	10–11	0	3	0	3	25	5	50	8	75	11	75	20

TABLE III.

Comparison of the Growth Capacity of the Two Sets of Infants.

Group.	Weight.	Increment from 3rd day.		Capacity.	
		Placenta.	Normal.	Placenta.	Normal.
	lbs.	*per cent*	*per cent*	*per cent*	*per cent*
A.	5–6	15.6	10.0	2.836	1.818
B.	6–7	12.0	9.1	1.846	1.400
C.	7–8	9.6	9.0	1.280	1.200
D.	8–9	7.8	7.2	0.918	0.847
E.	9–10	3.5	4.3	0.369	0.453
F.	10–11	6.9	3.6	0.657	0.343

that unit weight is able to add on greater increments of matter, from day to day, than can unit weight of infants feeding on milk from mothers not ingesting this substance.

A large series of comparative measurements of the mammæ of women taking and not taking the desiccated placenta, combined

with a study of the time of onset of full milk production, failed to show either hypertrophy of the gland or an increased milk production on the part of those women ingesting the placenta material. In view of these facts, and having in addition evidence that the increased food value of the milk produced during the administration of the dried placenta cannot compensate for the increased growth of itself,[2, 3] and moreover since but 30 grains, or less than a gram, of the dried material was fed each day, a quantity so small as to be negligible as matter from which new tissue can be built, we conclude that there must be contained in the desiccated placenta some substance, or substances, capable of passing through the maternal organism with at least a part of its activity retained. Being secreted by the mammary glands, it is passed on to the infant in the milk, there acting as stimuli to growth. It is not illogical to suppose that these substances in the placenta *in utero* may play an important part in the growth of embryo and fetus.

CONCLUSION.

The maternal ingestion of desiccated placenta produces an increase in the rate of growth and growth capacity of the breast-fed infants above that normally occurring. This is due to the presence in the placenta of some, as yet unidentified, growth-promoting substance.

ACTION OF ENZYMES ON HUMAN PLACENTA.

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The autolysis of human placenta has been the subject of several studies. That the placenta contained an autolytic enzyme was first shown by Mathes[1] in 1901. Ascoli,[2] Bergell and Liepmann,[3] and Bergell and Falk[4] also examined placenta for proteoclastic enzymes, with positive results, though the latter were unable to state whether the enzyme belonged to the tryptic or ereptic type. On the other hand, Lochhead and Cramer[5] were unable to find any true tryptic or ereptic enzymes. Löb and Higuchi[6] also failed to prove their existence. Within recent years exhaustive studies of the Abderhalden reaction have revealed the fact, however, that the proteoclastic enzyme of the blood, whether male or female, has a pronounced action upon placenta.[7] The action, however, of the digestive juices upon placenta does not appear to have been examined, though the medical literature contains many references to placental feeding. As the placenta and placental products are of high importance in many theories of the etiology of the toxic disturbances of pregnancy, it appeared important to us to undertake such a study. Moreover, our own experimental work was leading us in the direction of placental feeding and it was essential for us to be certain of the digestibility of our product. We have consequently made a short study of the action of the digestive enzymes upon our placenta preparation, and then sup-

[1] Mathes, P., *Centr. Gynäk.*, 1901, xxv, 1885.

[2] Ascoli, A., *Z. physiol. Chem.*, 1902, xxxvi, 498.

[3] Bergell, P., and Liepmann, W., *Münch. med. Woch.*, 1905, lii, 2211.

[4] Bergell, P., and Falk, E., *Münch. med. Woch.*, 1908, lv, 2217.

[5] Lochhead, J., and Cramer, W., *Proc. Roy. Soc., Series B*, 1908, lxxx, 263.

[6] Löb, W., and Higuchi, S., *Biochem. Z.*, 1909, xxii, 316.

[7] Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377.

plemented our experiments *in vitro* with a comparative digestibility trial. We may say at once that the placenta is readily attacked by pepsin, trypsin, and erepsin. It is also readily hydrolyzed by weak acids and alkalies. We have studied its digestibility in the dog by comparing it with meat, with which it has almost the same nitrogen content. Its digestibility, under the same conditions, on a protein and carbohydrate diet, is about the same as lean meat.

EXPERIMENTAL.

The placental substrate was prepared according to the directions of Harding and Fort.[8] The pepsin was a preparation of Parke Davis and Company; trypsin was prepared according to the directions of Cole[9] from sheep pancreas, and the erepsin

TABLE I.

Action of Pepsin.

No. of flask.	Enzyme solution.	0.4 per cent HCl.	Water.	Placenta.	Time of digestion.	0.05 N NaOH per 100 cc.	Hydrolysis.	Enzymatic acceleration.	Remarks.
	cc.	*cc.*	*cc.*	*gm.*	*hrs.*	*cc.*	*per cent*	*per cent*	
1	2	5	33	0.3926	24	9.40	9.70	6.96	Digestion practically complete in 24 hrs. Acid exerts a slight hydrolytic effect.
2	2	5	33	0.3927	24	5.96	2.74		
3	2	5	33	—	24	4.60	—		
1	2	5	33	0.3918	24	9.60	9.88	7.18	
2	2	5	33	0.3933	24	6.08	2.71		
3	2	5	33	—	24	4.72	—		
1	2	5	33	0.3931	48	9.80	9.83	7.08	
2	2	5	33	0.3911	48	6.00	2.75		
3	2	5	33	—	48	4.64	—		
1	2	5	33	0.3941	96	10.28	11.16	8.66	
2	2	5	33	0.3925	96	5.96	2.50		
3	2	5	33	—	96	4.72	—		
1	2	5	33	0.3925	96	10.20	10.90	8.24	
2	2	5	33	0.3927	96	6.12	2.66		
3	2	5	33	—	96	4.80	—		

[8] Harding, V. J., and Fort, C. A., *J. Biol. Chem.*, 1918, xxxv, 29.

[9] Cole, S. W., Practical physiological chemistry, Cambridge, 3rd edition, 1913, 94.

from pig intestine by simple extraction with distilled water, according to Cohnheim.[10] As a method of experimental procedure, we took three flasks and placed in them the following: (1) enzyme + acid or alkali + placenta; (2) enzyme (boiled) + acid or alkali + placenta; (3) enzyme + acid or alkali. In the protocols the number of the flask will refer to the flask as just described. The flasks were sterilized before use, and toluene

TABLE II.

Action of Trypsin.

No. of flask.	Enzyme solution.	0.4 per cent Na_2CO_3.	Water.	Placenta.	Time of digestion.	0.05 N NaOH per 100 cc.	Hydrolysis.	Enzymatic acceleration.	Remarks.
	cc.	*cc.*	*cc.*	*gm.*	*hrs.*	*cc.*	*per cent*	*per cent*	
1	5	10	25	0.3918	24	40.96	49.58	30.68	Digestion, as in the case of pepsin, is complete in 24 hrs. Sodium carbonate exerts a strong hydrolytic effect. All the placenta was dissolved at the end of the experiment.
2	5	10	25	0.3913	24	25.80	18.90		
3	5	10	25	—	24	16.48	—		
1	5	10	25	0.3917	24	41.48	50.33	32.20	
2	5	10	25	0.3924	24	25.60	18.12		
3	5	10	25	—	24	16.48	—		
1	5	10	25	0.3923	48	42.12	51.55	30.04	
2	5	10	25	0.3934	48	27.30	21.50		
3	5	10	25	—	48	16.64	—		
1	5	10	25	0.3954	48	42.20	51.02	29.68	
2	5	10	25	0.3940	48	27.30	21.34		
3	5	10	25	—	48	16.72	—		
1	5	10	25	0.3940	96	42.32	51.57	32.44	
2	5	10	25	0.3936	96	26.20	19.45		
3	5	10	25	—	96	16.72	—		
1	5	10	25	0.3951	96	42.62	52.02	31.45	
2	5	10	25	0.3928	96	29.60	20.56		
3	5	10	25	—	96	16.72	—		

was added as a preservative during the experiment. The flasks and contents were stoppered with absorbent cotton and incubated at a temperature of 37.5° for a definite number of hours. They were then removed from the incubator, and the enzyme was destroyed by rapidly bringing the contents of the flask to a boil and then cooling. The solution was then diluted to 100 cc., fil-

[10] Cohnheim, O., Enzymes, New York, 1912, 4.

tered, and the extent of proteoclasis in 25 cc. determined by the Sörensen[11] method, phenolphthalein being used as an indicator.

The results were calculated as the percentage hydrolysis of the amino nitrogen of the fully hydrolyzed placenta. The nitrogen content of the placenta was determined by the Kjeldahl method and found to be 12.46 per cent. According to Harding and Fort,[8] the amino-acid nitrogen (free and combined) of placenta

TABLE III.

Action of Erepsin.

No. of flask.	Enzyme solution.	0.4 per cent Na_2CO_3.	Water.	Placenta.	Time of digestion.	0.05 N NaOH per 100 cc.	Hydrolysis.	Enzymatic acceleration.	Remarks.
	cc.	*cc.*	*cc.*	*gm.*	*hrs.*	*cc.*	*per cent*	*per cent*	
1	5	0.5	44.5	0.3657	24	7.20	13.890	13.890	Digestion practically complete in 24 hrs. With the smaller amount of sodium carbonate there is no hydrolytic effect due to that cause.
2	5	0.5	44.5	0.3905	24	4.05	—		
3	5	0.5	44.5	—	24	4.00	—		
1	5	0.5	44.5	0.3791	24	7.50	14.358	14.358	
2	5	0.5	44.5	0.3897	24	4.05	—		
3	5	0.5	44.5	—	24	4.05	—		
1	5	0.5	44.5	0.3876	48	7.90	14.334	14.334	
2	5	0.5	44.5	0.3879	48	3.95	—		
3	5	0.5	44.5	—	48	4.40	—		
1	5	0.5	44.5	0.3906	48	8.05	14.473	14.473	
2	5	0.5	44.5	0.3871	48	4.05	—		
3	5	0.5	44.5	—	48	4.50	—		
1	5	0.5	44.5	0.3994	96	8.10	14.504	14.504	
2	5	0.5	44.5	0.3944	96	4.05	—		
3	5	0.5	44.5	—	96	4.45	—		
1	5	0.5	44.5	0.3924	96	8.00	14.562	14.562	
2	5	0.5	44.5	0.3868	96	4.10	—		
3	5	0.5	44.5	—	96	4.40	—		

is 70.8 per cent of the total nitrogen present. Therefore the percentage of amino-acid nitrogen in the placenta preparation was 8.82. The percentage hydrolyses were calculated on this figure.

In the comparative digestibility trial *in vivo* a diet of potato and lean beef was supplied to a puppy. The meat was freed as far as possible from fat and connective tissue, minced, and ex-

[11] Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

tracted with water. It was then coagulated, air-dried, and ground in a mill to a fine powder, thus resembling in its preparation and texture the placental powder. 15 gm. of meat were given per day, and the feeding was continued for 5 days. The nitrogen excreted in the urine was determined each day. A second period of 5 days then followed, with 15 gm. of placental powder

TABLE IV.

Puppy.	Nitrogen excretion per 24 hours.	
	Meat.	Placenta.
	gm.	*gm.*
W.	1.032	1.615
	1.689	1.503
	1.176	1.908
	1.153	1.764
	1.274	1.433
Average...	1.265	1.665
G.	0.881	1.241
	1.236	1.248
	1.172	1.319
	1.180	1.122
	1.293	1.193
Average...	1.152	1.225
N.	1.526	1.637
	1.512	1.758
	1.606	1.541
	1.602	1.474
		1.496
Average...	1.561	1.581

replacing the beef. The nitrogen content of the two is identical; both had been extracted with water and were of the same physical texture. The nitrogen excretion per day was again determined in the second period, and was found to be always slightly higher than the nitrogen of the first period. Hence it was concluded that the digestibility of placental powder is about the same as lean meat, under the same conditions. No ill effects were no-

ticed on the animals, who devoured the rations greedily, and who also showed increase in weight during the experimental period.

15 gm. of meat or placental powder, 250 gm. of potato, 35 gm. of cornstarch, or dextrin, 5 gm. of cane sugar, were supplied each day to a puppy of about 3 to 4 months. Nitrogen content of either diet was 2.0 gm.

SUMMARY.

Normal human placenta is readily hydrolyzed by pepsin, trypsin, and erepsin, and is readily and easily digested by the dog.

THE MEASUREMENT OF THE ACIDITY OF BREAD.

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The degree of acidity is of importance in bread making in that it determines the physical state of the gluten, influences the growth and the activity of the yeast, and controls the growth of many other microorganisms, such as the rope-producing bacillus *Bacillus mesentericus*. The growth of yeast and other microorganisms in turn influences the acidity of dough and of bread. A colorimetric method for measuring the relative acidity of bread is therefore suggested.

The effect of the hydrogen ion concentration[1] upon the hydration, the viscosity, and the degree of dissolution of gluten has been the subject of researches by Wood, Wood and Hardy, Olson, Stockholm, Upson and Calvin, Gortner and Doherty, and numerous other investigators. These authors have made observations which experiments soon to be published from this laboratory have confirmed and at certain points extended. A comparison of the results obtained from the study of gluten with such baking experiments as those of Jessen-Hansen and others seems to indicate that there is an optimum hydrogen ion con-

[1] The degree of acidity is best measured by the electrometric or colorimetric determination of the concentration in hydrogen ions, commonly expressed as pH.

centration for the baking of bread. As this paper goes to press the investigation of Landenberger and Morse can be cited in further support of this view.

The hydrogen ion concentration of bread is related to the original acidity and to the acid-combining power of the cereals used, to the amount of yeast, and in that the activity of yeast varies with the acidity of the medium and modifies it through the acid products of its own metabolism, to the conditions of fermentation.

Further, sufficient acidity effectively checks the growth of many other microorganisms. In general this is an important matter in the technology of fermentation; specifically in bread making it is involved in the problem of the prevention of rope. At the present time the last factor is of serious economic importance for we have found[2] that a large variation in the acidity of bread results from the changing formulas and different substances which the bakers are at present obliged to use. This is illustrated by the data collected in Table II of the variation in hydrogen ion concentration of the loaves of the largest Boston bakeries. Usually the wheat substitutes tend to reduce the acidity of the loaf. We have, however, also found that beyond a hydrogen ion concentration of 10^{-5}N (pH 5) *Bacillus mesentericus*, which seems to be the common cause of rope, cannot develop in bread, and that its development is inhibited as the hydrogen ion concentration approaches 10^{-5}N.

It has therefore seemed desirable to determine the hydrogen ion concentration of bread in a considerable number of cases, and to find a method of making this measurement which shall be at once trustworthy and sufficiently simple.

At the outset this problem is complicated by uncertainty of definition, for it is not clear what meaning is to be attached to the phrase, *the hydrogen ion concentration of bread*, and in any case this concentration cannot be directly measured so as to give absolute rather than relative values of known accuracy. Furthermore there is a considerable difference in the hydrogen ion concentration measurements, whether with concentration cell or with indicators, of aqueous extracts of bread on the one hand, and of suspensions on the other. Nevertheless experience has

[2] See Bibliography, Cohn, Wolbach, and Henderson, and Henderson.

proved that consistent results, which are quite satisfactory for practical purposes and which may be provisionally expressed in terms of hydrogen ion concentration, are not difficult to obtain. For the present the more exact definition of the question may accordingly be postponed.

When a drop of the ordinary indicator solution of methyl red falls upon a slice of bread it assumes a color which may vary, according to the acidity of the loaf, from orange to red. If the loaf is ropy, the color will probably be yellow, as a result of the alkali produced in the metabolism of *Bacillus mesentericus*. Baking experiments upon dough of known but graduated acidities show that the range from orange to red corresponds to initial values of pH ranging from approximately 6.0 to 4.5.

TABLE I.

Effect of Lactic Acid upon Hydrogen Ion Concentration of Dough and of Bread.

Experiment 744.	0.5 N lactic acid added to 300 gm. of flour.	pH		
		Immediately upon mixing dough.	After 4 hrs. fermentation just before baking.	Bread after baking.
	cc.			
1	0		5.30	5.38
2	1.0		5.24	
3	1.6	5.55		
4	2.4			
5	3.2		4.98	
6	4.0	5.20	4.94	4.98

An experiment in which the acidity of the dough and of the resulting bread was progressively increased by the addition of lactic acid is reported in Table I. The resulting loaves clearly showed the gradient in color of methyl red.

This result has led us to make parallel observations on the hydrogen ion concentrations of aqueous suspensions[3] of nu-

[3] The suspensions of bread were made in the following manner: A fresh cut was made in the loaf and a 5 gm. sample was then cut from the center. This was ground with 25 cc. of distilled water in a small mortar until the bread was well broken. With the exception of the largest pieces, all the finely broken material was poured into the concentration cell.

merous loaves of bread, and upon the color of these loaves when methyl red is added to them. The reaction as indicated by methyl red is expressed on an arbitrary scale ranging from 1 (most acid) to 7 (most alkaline).

These measurements (Table II) seem to afford sufficient evidence that the hydrogen ion concentrations of suspensions of bread run closely parallel with the colors produced on the cut surface by the addition of methyl red. The accompanying figure

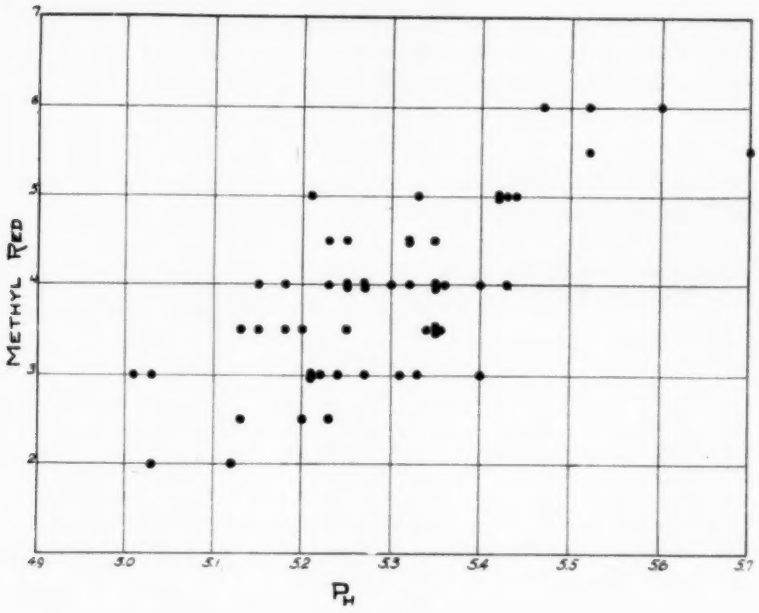

FIG. 1.

indicates the measure of agreement of the two methods. Accordingly, it seems possible to estimate what may not improperly be called the hydrogen ion concentration of bread with the help of this indicator.

The method to be employed is as follows: The loaf is cut cleanly, and upon a point near the center of the loaf four drops of a 0.02 per cent solution of the indicator in 60 per cent alcohol are allowed to fall. After waiting 5 minutes the color is observed.

TABLE II.

Electrometric and Colorimetric Determination of the Hydrogen Ion Concentration of Bread.

Baker.	Trade name of bread.	Date of measurement.	pH of bread.	No. of color plate.
		1918		
Hathaway and Sons.	Subway.	Aug. 7	5.01	3
Ward Baking Co.	Mother Hubbard.	" 8	5.03	2
Hathaway.	Subway.	" 7	5.03	3
Ward.	Mother Hubbard.	" 9	5.12	2
"	" "	" 5	5.13	2.5
"	" "	" 7	5.13	3.5
"	Tip Top.	" 7	5.15	3.5
Hathaway.	Subway.	" 7	5.15	4
Ward.	Tip Top.	" 7	5.18	3.5
Hathaway.	Subway.	" 8	5.18	4
Ward.	Oaten Loaf.	" 9	5.20	2.5
"	Daintymaid.	" 7	5.20	3.5
"	Oaten Loaf.	" 8	5.21	3
"	Daintymaid.	" 8	5.21	3
Hathaway.	Vienna.	" 7	5.21	5
Ward.	Daintymaid.	" 6	5.22	3
"	Mother Hubbard.	" 8*	5.23	2.5
Hathaway.	Subway.	" 5	5.23	4.5
Ward.	Tip Top.	" 7	5.23	4
"	Daintymaid.	" 9	5.24	3
"	Mother Hubbard.	" 9*	5.25	3.5
"	Tip Top.	" 8	5.25	4
Hathaway.	Subway.	" 9	5.25	4
General Baking Co.	Mrs. Walker's Prize.	" 8	5.25	4.5
Ward.	Oaten Loaf.	" 8*	5.27	3
"	Tip Top.	" 5	5.27	4
"	Oaten Loaf.	" 9*	5.27	4
Hathaway.	Subway.	" 8*	5.30	4
Ward.	Daintymaid.	" 8*	5.31	3
Hathaway.	Subway.	" 7	5.32	4
General.	Mrs. Walker's.	" 7	5.32	4.5
Hathaway.	Subway.	" 9*	5.32	4.5
General.	Mrs. Walker's.	" 7	5.33	3
"	" "	" 5	5.33	5
Ward.	Daintymaid.	" 6	5.34	3.5
"	Tip Top.	" 8*	5.35	3.5
"	Daintymaid.	" 9*	5.35	3.5
"	"	" 6	5.35	3.5
General.	Mrs. Walker's.	" 9*	5.35	4

TABLE II—*Concluded.*

Baker.	Trade name of bread.	Date of measurement.	pH of bread.	No. of color plate.
		1918		
Ward.	Tip Top.	Aug. 6	5.35	4
General.	Mrs. Walker's.	" 9	5.35	4.5
Hathaway.	Subway.	" 6	5.36	4
Ward.	Daintymaid.	" 6—*	5.40	3
"	Tip Top.	" 9	5.40	4
General.	Mrs. Walker's.	" 5—*	5.42	5
"	" "	" 8*	5.42	5
"	" "	" 6	5.43	5
Ward.	Mother Hubbard.	" 6	5.43	4
Spindler's Bakery.		" 7	5.44	5
Anastos and Chakalis.		" 6	5.47	6
" " "		" 6	5.52	6
Hathaway.	Cream Loaf.	" 7	5.52	5.5
Anastos and Chakalis.		" 6*	5.60	6
Ward.	Tip Top.	" 9*	5.70	5.5

*Determination made on the day after the loaves were bought and brought into the laboratory.

By comparison with a color chart or with a loaf of bread of known acidity, the hydrogen ion concentration of a loaf of bread may be estimated with an error no greater than that indicated by the above determinations.

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A QUANTITATIVE STUDY OF THE EVAPORATION OF BLOOD SERUM.

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A need for the dried solids of blood serum in immediately soluble form has led to a study of methods of low temperature evaporation of protein solutions. The method adopted may be of use in other instances.

The blood freshly collected at the abatoir was allowed to clot undisturbed in cylindrical vessels of several gallons capacity. After separating with a thin knife blade the strands of the clot from the sides of the vessel, the serum was syphoned into bottles and promptly centrifuged to remove the small quantity of corpuscles not held by the clot. This obviates laking of the blood, and yields a perfectly clear, amber colored liquid.

The serum containing somewhat less than 9 per cent of total solids, to gain any considerable amount of dried residue, demands the evaporation of a large amount of water. This evaporation may be carried out in a current of warmed air at atmospheric pressure, the exposed surface of the serum being increased by placing the liquid in large, salt-mouth bottles arranged horizontally and rotated. The rate of evaporation so induced is rather low, and this prolonged warming tends to denature the proteins, rendering them less soluble.

The obvious alternative is to carry out the process under reduced pressure. This method was applied with excellent results in the way now to be described, which has the especial advantage that the process may easily be followed in a quantitative manner.

An ordinary distilling flask of at least 1 liter capacity was supplied with perhaps 100 gm. of rather large glass pearls, and weighed. It was then immersed to about the level of the side tube in water of the desired temperature. In our experiments, this temperature did not exceed 50°C. A dropping funnel was introduced into the flask, the end of the stem extending well into the bulb. The side tube of the flask was connected with an efficient condenser, arranged vertically and emptying into a previously weighed bottle of size suitable for trapping the evaporated and recondensed water. After this followed in succession a small, weighed calcium chloride tower, a manometer, and the vacuum pump. The trap bottle and the calcium chloride tower were immersed in ice water, and ice water flowed through the condenser jacket.

The apparatus being evacuated to a pressure of 1 cm. of mercury, or less, the liquid serum was allowed to enter slowly through the funnel, and, as nearly as possible, at the rate at which evaporation proceeded. So treated, the serum foams largely and leaves a friable product that adheres rather loosely to the walls of the flask.

When the desired amount of liquid had been evaporated, the cold water of the condenser jacket was replaced by warm water and air was slowly admitted through the funnel. This was followed by reevacuation. As the evaporation neared completion, the temperature of the bath was raised somewhat. When the serum was dry, the apparatus was disassembled and the parts previously weighed were dried and reweighed.

Through giving the flask a vigorous rotary motion, the pearls were made to loosen and pulverize the product, which was separated from them by pouring onto a sieve of proper mesh.

The following data of preparations show the precision of which the procedure is readily capable. The density of the serum used in the preparations was $d_4^{25} = 1.0234$. The bath temperature was between 45 and 50°.

Quantitative Preparation of Dried Serum.

	gm.
Weight of serum delivered to dropping funnel.....	= 102.02
" " " collected in trap bottle.....	= 90.30
" " " " " tower.....	= 2.60
" " solid residue in flask.....	= 9.11
	102.01

The trapped water was perfectly clear and colorless. Its specific conductance was approximately 7.7×10^{-5}; it was thus nearly free of electrolytes. It had a slight ammoniacal odor and was faintly alkaline. The pH was 8.1. The pH of the original serum, which had not been adjusted to a particular CO_2 tension, was 7.65, and its conductance 1.22×10^{-2}. Redissolved in carbon dioxide-free water in its original concentration, the reaction of the preparation was pH 9.4.

The solids of the serum thus constituted 8.93 per cent of the whole. Two different samples of serum from the same source proved closely constant in this respect, as the following data show.

Experiment No.	Liquid serum.	Residue.	Solids in serum.
	gm.	*gm.*	*per cent*
770	102.02	9.10	8.92
771	102.02	9.16	8.98
772	102.02	9.11	8.93

The powdered product is of light yellow color and of faint odor. It dissolves readily in distilled water to a solution that is but slightly turbid. Determination of its nitrogen content by the Kjeldahl method gave the following results:

Nitrogen.
per cent
11.66
11.83
Average, 11.75

According to Michaelis and Davidsohn, the isoelectric point of serum albumin appears at about pH 4.7. The electrometric titration of serum as reported by McClendon and confirmed by determinations in this laboratory shows that when approximately 0.06 cc. of normal hydrochloric acid is added to each cc. of serum, the pH is 4.5. Serum so treated and evaporated leaves, as would be expected, a product more friable and less soluble in water than that from the untreated serum. Such a preparation is presumably near the isoelectric point of the proteins, and readily dissolves upon the addition of acid or of alkali.

Data of a preparation from serum treated with less acid than the above requirement are as follows:

Preparation of Acidified Serum.

	gm.
Weight of serum taken	102.02
" " normal hydrochloric acid added	4.08
	106.10
Weight of liquid water collected	78.88
" " water in drying tower	18.03
" " solid residue	9.13
	106.04

Before evaporation, the reaction of the acidified serum unadjusted to a definite carbon dioxide tension was pH 5.3. The redissolved preparation was slightly less acid, pH 5.5. In its behavior it lies between the preparation from unacidified serum and that from serum evaporated at its isoelectric point. It has the advantage of being more easily soluble than the latter, and, for some purposes, of having a less alkaline reaction than the former. The carbonates of the serum can thus conveniently be transformed into chlorides or into the salts of other acids.

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